INTERACTIONS OF THE GUT MICROBIOTA AND THE INNATE IMMUNE SYSTEM

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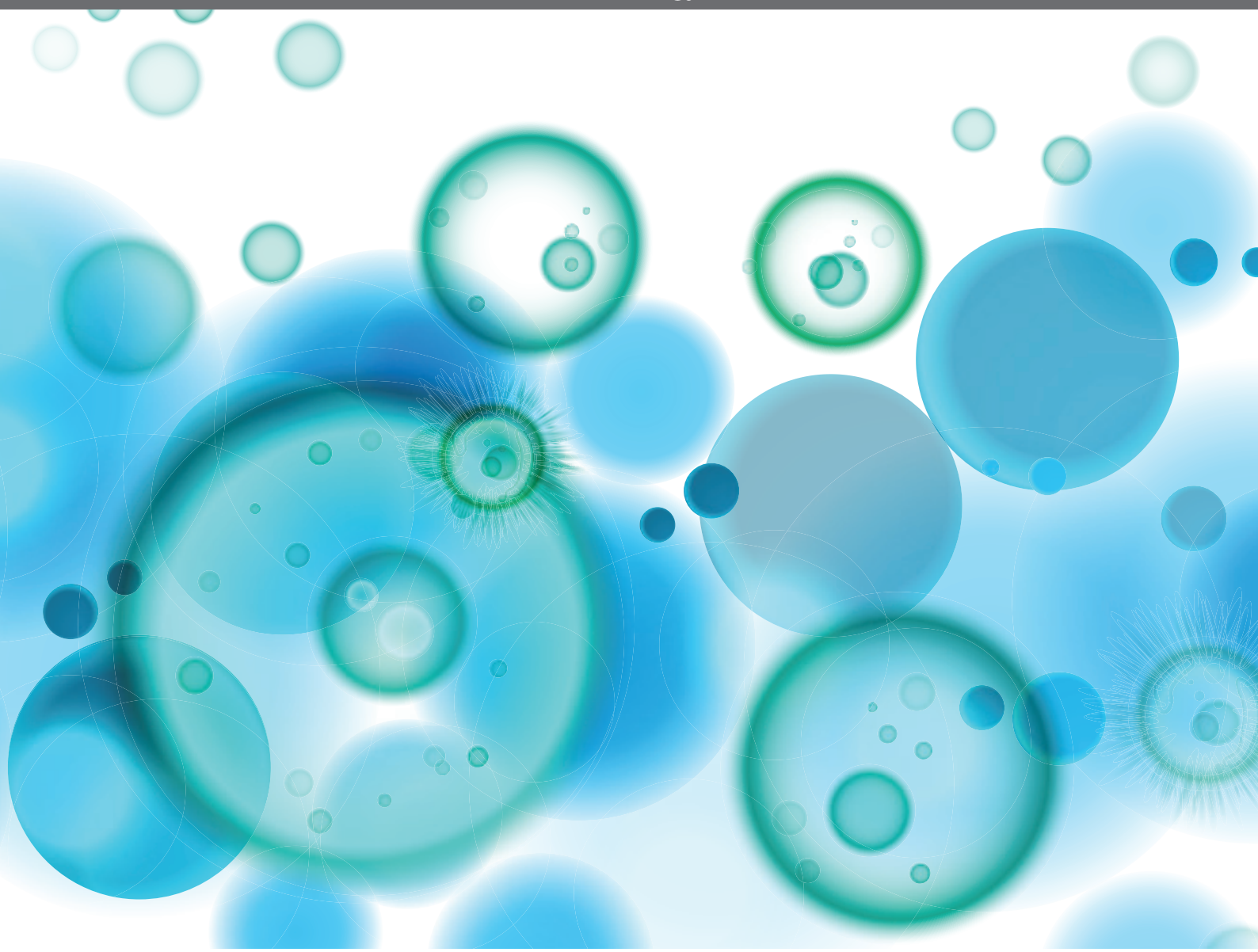

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INTERACTIONS OF THE GUT MICROBIOTA AND THE INNATE IMMUNE SYSTEM

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REVIEW
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Targeting the Gut Microbiota in Chagas Disease: What Do We Know so Far?

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Chagas disease (CD) is a tropical and still neglected disease caused by *Trypanosoma cruzi* that affects >8 million of people worldwide. Although limited, emerging data suggest that gut microbiota dysfunction may be a new mechanism underlying CD pathogenesis. *T. cruzi* infection leads to changes in the gut microbiota composition of vector insects, mice, and humans. Alterations in insect and mice microbiota due to *T. cruzi* have been associated with a decreased immune response against the parasite, influencing the establishment and progression of infection. Further, changes in the gut microbiota are linked with inflammatory and neuropsychiatric disorders, comorbid conditions in CD. Therefore, this review article critically analyses the current data on CD and the gut microbiota of insects, mice, and humans and discusses its importance for CD pathogenesis. An enhanced understanding of host microbiota will be critical for the development of alternative therapeutic approaches to target CD, such as gut microbiota-directed interventions.

Keywords: Chagas disease, *Trypanosoma cruzi*, gut microbiota, prebiotics, probiotics

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INTRODUCTION

Chagas disease (CD), also known as American trypanosomiasis, is a malady that affects >8 million people worldwide (Lidani et al., 2019) resulting in high socioeconomic burden to our society (Lee et al., 2013). Although it was discovered more than 100 years ago by Carlos Chagas and comes with a high health burden (Chagas, 1909; Lee et al., 2013), it continues to be a neglected disease (Schofield et al., 2006; Clayton, 2010). The flagellate protozoan *Trypanosoma cruzi* (*T. cruzi*) is the causative agent of CD, and it is primarily transmitted to humans and animals via insect vectors known as triatomines. CD is more prevalent and endemic in Latin American countries. However, more recently, other non-endemic areas, such as United States, Canada, Europe, Australia, and Japan are starting to be affected by CD due to increased immigration world-wide (Gascon et al., 2010; Jackson et al., 2014). It is thought that CD burden could be attenuated with disease control approaches, including vector control and treatment of the infection at an early stage (Schofield et al., 2006; Gascon et al., 2010).

Infection symptoms include inflammation in the gastrointestinal tract (GIT) and heart dysfunctions and may also include neurological and behavioral disturbances (Prata, 2001; Marchi and Gurgel, 2011; Ozaki et al., 2011; Vilar-Pereira et al., 2015; Pérez-Molina and Molina, 2018). Interestingly, recent evidence now suggests an involvement of other biological factors that may contribute to mechanisms underpinning CD pathophysiology. For instance, the role of the gut microbiota in CD has been reported by preclinical and clinical studies (Duarte et al., 2004, 2005; McCall et al., 2018; Robello et al., 2019; De Souza-Basqueira et al., 2020). Gut microbiota alterations have been found in triatomine insects, mice, and human hosts (Duarte et al., 2004, 2005; Garcia et al., 2007; Díaz et al., 2016; McCall et al., 2018; Robello et al., 2019). For example, alterations in the gut microbiota of triatomine insects, including changes in the Enterobacteriaceae and Nocardiaceae family as well as low CFU counts, may increase the susceptibility to infection by impairing immune response against *T. cruzi* (Castro et al., 2012; Vieira et al., 2016). It is possible that gut microbiota alterations induced by *T. cruzi* may aggravate the host's pathology due to modulation of the immune system (Díaz et al., 2016). However, the impact of the gut microbiota on CD's pathophysiology remains to be fully understood. In this review, we critically analyze the current data associating CD and the gut microbiota and the importance of this interaction for CD pathogenesis. An enhanced understanding of this relationship will be critical for the development of alternative therapeutic approaches for CD treatment.

CHAGAS DISEASE TRANSMISSION AND INFECTION CYCLE

The mode of CD transmission varies according to geography (Lidani et al., 2019). In Latin America, it is mainly transmitted via insect vectors known as triatomines, which are infected with the parasite after a blood meal from infected humans or other animals, according to the parasite's life cycle (**Figure 1**). In non-endemic places, blood transfusion, organ donation, congenital transmission during pregnancy, or via oral route with contaminated food and water are the main modes of CD transmission (Lidani et al., 2019). After infection with *T. cruzi* the disease can follow two distinct phases. In the acute phase, an increased parasitemia is observed. Although at this stage the disease is usually asymptomatic, there may be a few symptoms, such as fever, inflammation, tachycardia, fatigue, which can spontaneously disappear in most patients (Prata, 2001; Clayton, 2010; Pérez-Molina and Molina, 2018; Lidani et al., 2019). The chronic phase of the disease affects one third of the patients and begins with a latency period known as the indeterminate form of CD. This can persist for more than 30 years or throughout life unnoticed. After that phase, some patients can develop a symptomatic phase in which a decline in parasitemia and neurological, cardiac and digestive manifestations are observed as well as neuropsychiatric comorbidities and behavioral changes (Prata, 2001; Hueb and Loureiro, 2005; Clayton, 2010; Marchi and Gurgel, 2011; Ozaki et al., 2011).

THE GUT MICROBIOTA

The gut microbiota comprises a community of bacteria, archaea, fungi, and viruses that has co-evolved with their hosts over thousands of years to form intricate commensal relationships (Rinninella et al., 2019). The bacterial cell number present in the human gut has been estimated to be around 3.8×10^{13}, which is similar to our cell number in the body (Sender et al., 2016). Although the number of bacterial species is high, most of them belong to Firmicutes, Bacteroidetes, and Actinobacteria phyla (Falony et al., 2016). The gut microbiome interacts with host essential physiological processes such as modulation of immune system, metabolism, and neurotransmission, which ultimately coordinate host homeostasis (Keely, 2017). Regarding immunity, it is known that the gut microbiota modulates the development of $CD8^+$ T cells, lymphocytes with key roles in the control of *T. cruzi* (Martin and Tarleton, 2004; Acosta Rodríguez et al., 2019). Gut microbiota changes with antibiotic treatment were associated with altered cytokine response and T cell receptor (TCR) signaling in $CD8^+$ T cells (Gonzalez-Perez and Lamousé-Smith, 2017). Furthermore, certain strains of gut bacteria are able to induce $CD8^+$ T cells in the intestine, which is associated with enhanced immunity against *Listeria monocytogenes* and enhanced anti-tumor activity (Tanoue et al., 2019). The contribution of the gut microbiome to human health and disease continues to be unraveled. However, in the recent years, large-scale studies using emerging technologies in microbiome research, including 16S ribosomal RNA (rRNA) sequencing for taxonomic characterization and whole genome shotgun (WGS) metagenomic sequencing for genomic and metabolic functional analysis are accelerating the discovery of new links between the gut microbiome, human health, and disease (Kho and Lal, 2018).

The gut microbiota signals to their host using metabolic products, neurotransmitters, cytokines, and anti-microbial substances (Fischbach and Segre, 2016). On the microbiota side, one of the most studied mediators of this communication are bioactive molecules named short-chain fatty acids (SCFAs), which consist of bacterial-derived dietary fermentation products. The most common SCFA are acetate, propionate, and butyrate. SCFAs can modulate host cell functions by controlling gene transcription through epigenetic pathways, through the activation of "metabolite-sensing" G-protein coupled receptors (GPCRs) or indirectly via interactions with host's energetic metabolism and immune system (Wilson, 2005; Smith et al., 2013). Importantly, acetate, the most abundant SCFAs present in the blood circulation, influences cardiac function such as blood pressure and heart rate in mice via at least two modes: renin release in the juxtaglomerular apparatus and changes in vascular tone in the periphery (Onyszkiewicz et al., 2020). Furthermore, butyrate can inhibit histone-deacetylases (HDACs), affecting the gene expression of $CD8^+$ cytotoxic T cells (CTLs) and causes an increase in the expression of interferon-γ (IFN-γ) and granzyme B in this cell population (Luu et al., 2018).

Another key mediator factor between the gut microbiota and their hosts is the immune system. The gut microbiota is known to direct the immune system maturation, development,

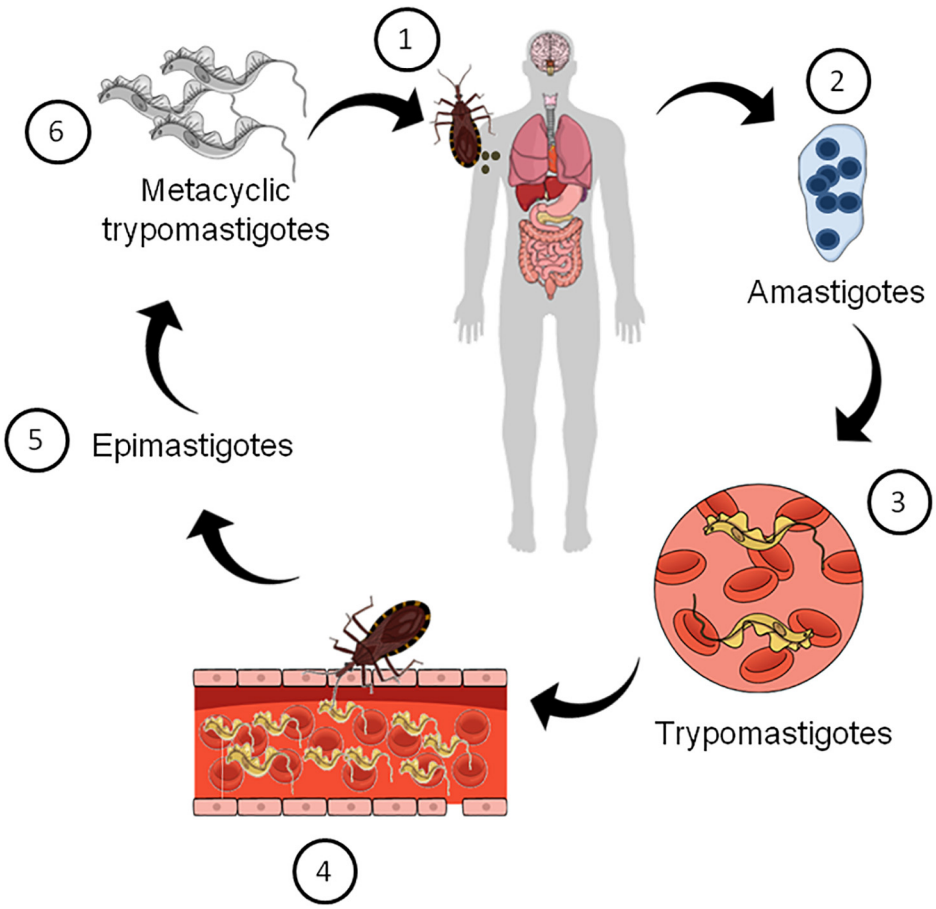

FIGURE 1 | Schematic summarizing the life cycle of *Trypanosoma cruzi*. (1) Triatomine bugs take a blood meal and passes *metacyclic trypomastigotes* in their feces, which then enter the host via bite wounds or mucosal membranes. Metacyclic trypomastigotes now invade host cells (not shown) and become *amastigotes*, which multiple inside the infected cells (2). Amastigotes then transform into *trypomastigotes*, which then cause cell lysis and their release into the bloodstream (3). Triatomine bugs become infected when they feed on an infected mammalian host (4). Inside the insect vector, trypomastigotes now transform into *epimastigotes* in the insect gut (5). Finally, epimastigotes transform into infective *metacyclic trypomastigotes* (6), which are released in the feces and the cycle re-starts.

and response. Specific gut microorganisms named segmented filamentous bacterium, non-culturable Clostridia-related species, guide the development of hosts IL-17 (Th17) and IFNγ (Th1) T cells in the gut mucosal immune system of rodents (Gaboriau-Routhiau et al., 2009; Hooper et al., 2012). Further, colonization of germ-free (GF) mice (mice born and raised without microbiome) with commensal microbiota induces IgA in intestinal dendritic cells, further affecting hosts defense against non-pathogenic and pathogenic microorganisms. Microbial signaling to host immune system is also facilitated by microbial metabolites and by bacterial products such as SCFAs and polysaccharide (PSA), among others (Kim et al., 2013; Macia et al., 2015). From the host side, signals are sent via hormones, cytokines, anti-microbial products, which will further change gut microbial communities' structure and function (Fischbach and Segre, 2016). Thus, it is not surprising that such intricate relationship between the gut microbiota and their hosts are implicated in many diseases, ranging from inflammatory and cardiovascular diseases, metabolic, psychiatric, and neurological conditions. The bidirectional communication between the microbiota and other physiological systems of the body, such as the nervous, endocrine, and immune system collectively form the *microbiota-gut-brain axis* (Cryan et al., 2019). This constant crosstalk allows the host to maintain homeostasis of essential physiological processes, such as neurotransmission, appetite, neuroprotection, neurogenesis, which ultimately coordinate behavior. Data now available demonstrate that dysfuncion in the gut microbiota homeostasis play a role in many chronic inflammatory, neuroinflammatory, and neuropsychiatric disorders (Burokas et al., 2015; Sherwin et al., 2016; Sandhu et al., 2017). However, the role of the gut-microbiota-brain axis in CD remains largely unknown. Therefore, the exploring its role could provide new insights into CD pathophysiology and treatment.

THE GUT MICROBIOTA IN CHAGAS DISEASE

Microorganisms that inhabit the gut of vector insects have a pivotal role in the modulation of vector competence, which is "the ability to acquire, maintain, and transmit pathogens" (Lane, 1994). These microbes can interfere with vector competence either directly or indirectly (Dillon and Dillon, 2004; Azambuja et al., 2005; Garcia et al., 2007, 2010; Cirimotich et al., 2011; Weiss and Aksoy, 2011). In the first case, they interact with parasites and compete for resources present in the triatomine gut. In the second case, the triatomine gut microbiota can trigger anti-parasitic mechanisms and immune responses against the parasite, modulating parasite transmission (Dillon and Dillon, 2004; Azambuja et al., 2005; Garcia et al., 2007, 2010; Cirimotich et al., 2011; Weiss and Aksoy, 2011). A study that characterized triatomine bacterial communities demonstrated that *T. cruzi* infection changes the gut microbiota of these insects and it depends on "the intrinsic qualities" of the parasite itself, the insect vector and the gut microbiota of the host (Díaz et al., 2016). In the laboratory setting, the triatomine gut microbiota is characterized by low diversity of microbial population and dominance of one or few genera and specificity of bacteria to some triatomine hosts, which means that some genera of bacteria are found in specific hosts but not all host species (Díaz et al., 2016).

GUT MICROBIOTA ALTERATIONS IN CLINICAL AND EXPERIMENTAL CD: A NEW PLAYER IN CD PATHOGENESIS?

Vector–Parasite–Microbiota Interaction in Mice and Humans

Over the years, studies focusing primarily on the parasite-vector and parasite-host cell interaction have been performed, and much insight regarding *T. cruzi* infection establishment and disease progression was gained following this approach (De Souza et al., 2010; De Oliveira et al., 2018). Nonetheless, given the crucial role the microbiota has to the host itself, the studies herein discussed focused on a tripartite interaction known as the "vector–parasite–microbiota interaction," which is an approach that considers the gut microbiota of the host as relevant to disease establishment and progression (Díaz et al., 2016). The idea that gut microbiota was somehow linked to CD was previously hypothesized in a series of studies using GF mice. An early study comparing the impact of acute Y strain of *T. cruzi* infection on GF mice and conventional mice demonstrated that the absence of gut microbiota in the GF mice leads to a much more severe course of infection (Silva et al., 1987). A possible reason for these outcomes is that, as showed in another study, acutely infected mice also had an impaired cellular and humoral-mediated immune response against the parasite, as observed by lowered levels of IFN-γ, TNF-α, nitric oxide (NO), and antibodies specifically generated against *T. cruzi* antigens (anti-*T.cruzi* IgG1 and IgG2a) (Duarte et al., 2004). These findings are in keeping with the fact that the microbiome educates and shapes the immune system (Lee and Mazmanian, 2010; Hooper et al., 2012).

Consequently, it is expected that GF mice have an immature immune system and therefore display an altered immune response to an immune challenge. It should be noted that these studies do not offer a causal association between the gut microbiota and CD, but rather demonstrate the importance of the gut microbiota on priming the immune response in the context of parasitic diseases. Whether specific members of the microbial community contribute to the modulation of CD remains to be determined.

In a preliminary attempt to answer this question, a sequence of studies investigated the role of specific gut bacteria on mediating immunomodulatory effects in the host following acute exposure to *T. cruzi* (Silva et al., 1987; Duarte et al., 2004). In a study, GF Swiss mice received a single intragastric and individual injection of *Escherichia coli*, *Enterococcus faecalis*, *Bacteroides vulgatus*, or *Peptostreptococcus* sp. 10 days prior to infection with 5×10^3 *T. cruzi trypomastigotes* (Y strain) (Please refer glossary). The authors found that *gnotobiotic mice* had increased survival rates when compared to control GF mice (Duarte et al., 2005), which tend to die earlier after infection (Silva et al., 1987). Conversely, a separate study demonstrated that monocolonization with *Bacteroides fragilis*, *Clostridium* sp., and even *Peptostreptococcus* sp. were associated with earlier mortality in acute experimental CD (Barros et al., 1992). The results for *Bacteroides fragilis* are undoubtedly surprising, since polysaccharide A (PSA) derived from this bacterium has modulatory effects on the immune system, driving its maturation and balance of Th1/Th2 responses in mice (Mazmanian et al., 2005), which would be ideal for *T. cruzi* infection resolution. The reasons for these apparently contradicting data are hitherto undefined, buy may be due to a more Th2-associated response, which consequently promotes parasite's persistence in the host.

Interestingly, increased levels of NO were only observed in *E. coli* and *Peptostreptococcus* sp.-associated mice, while the lowest and highest production of IgG1 and IgG2a levels, respectively, were only shown in *Peptostreptococcus* sp.-associated mice. Furthermore, only mice that received *E. faecalis* had a rise in IL-10 in cultured splenocytes. These findings suggest that some of the observed immunomodulatory effects depends on certain bacterial species and cannot be generalized (Duarte et al., 2005). In fact, immunomodulation is a well recognized feature of commensal gut bacteria, contributing not only to mucosal immunity, but also to immune tolerance (Donaldson et al., 2015; Libertucci and Young, 2019). Collectively, these data demonstrate that colonization of the gut with specific bacteria strains normalizes the immune function, allowing the host to respond to *T. cruzi*.

The studies so far reported only showed that the gut microbiota has a role in immune homeostasis, which is important for the resolution of acute infection with parasites in general. However, a recent study using male C3H/HeJ mice infected with a luciferase-expressing *T. cruzi* strain (CL Brener) demonstrated a more direct association between *T. cruzi* infection and gut microbiota alterations. In this study, the authors sampled fecal pellets twice a week during the acute

phase and every 2–3 weeks during the chronic phase. Using 16s rRNA sequencing it became evident that *T. cruzi* infection was associated with changes in *Bacteroidales* and *Clostridiales* order, which belong to *Bacteroidetes* and *Firmicutes* phyla. Further, host fecal metabolic status was assessed using ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) and revealed alterations in fatty acids and bile acids metabolism in mice infected with the parasite (McCall et al., 2018).

More pronounced differences between groups were found at 21-days post-infection. Linoleic and linolenic acids are fatty acids metabolized by gut bacteria, including by members of the family *Ruminococcaceae* and *Lachnospiraceae* (phylum *Firmicutes*) that are present in the mice cecum and feces (Gu et al., 2013), into conjugated linoleic and linolenic acid and other derivative molecules, such as vaccenic acid (Zhang and Davies, 2016). These metabolites have been associated with anti-inflammatory response in other disease models such as animal models of colitis (Bassaganya-Riera et al., 2004; Miyamoto et al., 2015) and colorectal cancer (Evans et al., 2010). For instance, in colitis animal models, linoleic acids can act locally by downregulating the TNF-α receptor (Miyamoto et al., 2015) and TNF-α expression (Bassaganya-Riera et al., 2004) and upregulating production of anti-inflammatory cytokine TGF-β in the colon (Bassaganya-Riera et al., 2004). Moreover, they decrease the infiltration of immune cells in the colon of mice with colorectal tumors, while increasing the number of regulatory T cells (Tregs) in the mesenteric lymph node (Evans et al., 2010).

Thus, a plausible possibility is that changes in the gut microbiome composition and consequently fecal metabolite alteration may favor *T. cruzi* survival through inducing an anti-inflammatory response in the host (McCall et al., 2018). However, whether these outcomes are relevant to humans with CD is still a matter of debate. Moreover, primary bile acids are produced by the host and further modified by the gut bacteria, including members of Clostridiales order or from the genus *Bifidobacterium* or *Lactobacillus*, to generate secondary bile acids (2BAs) such as deoxycholic acid. Changes in the metabolism of bile acids are associated with inflammation in the GIT (Gérard, 2013), but its role in CD remains poorly understood. Taken together, experimental CD leads to changes in bacteria that modulate fatty acids and bile acid metabolism in mice and these fecal microbiome and metabolome changes may be relevant for the persistence of *T. cruzi* in the host.

A recent study was conducted with twenty Bolivian children who were diagnosed with CD and treated with benznidazole, a CD first line treatment drug (Robello et al., 2019). Fecal samples were obtained before and after treatment and 16s rRNA sequencing was used to analyze the microbiota and uninfected subjects were used as controls. It was observed that the parasite induced changes not only in the gut microbiota of these individuals, but also in the skin microbiota. *T. cruzi* infection was associated with high amounts of fecal *Prevotella* (phylum Bacteroidetes), *Ruminococcaceae* and *Succinivibrio* (phylum Proteobacteria). In humans, *Prevotella* was associated with increased plasma levels of trimethylamine-*N*-oxide (TMAO), a molecule implicated in cardiovascular disease (Koeth et al., 2013). Overall, infected children had increased Firmicutes and lowered Bacteroides, despite variations in age and diet (Robello et al., 2019).

Another study employing next generation sequencing (NGS) to investigate the gut microbiota composition of one hundred and fourteen Brazilian individuals with different forms of chronic CD also confirmed that *T. cruzi* triggers human gut microbiota changes (De Souza-Basqueira et al., 2020). In this study, thirty patients had the cardiac form, eleven had the digestive form, thirty-two had the indeterminate form and thirty-one were healthy individuals. The authors found lowered levels of Verrucomicrobia phylum as well as decrease in Veillonellaceae family (phylum Firmicutes) and *Dialister* genera (phylum Firmicutes) in patients with cardiac CD. Indeterminate CD patients had lowered Bacteroidaceae family (phylum Bacteroidetes), specifically Bacteroides genera when compared to controls. Patients with digestive form of CD and megacolon had reduction in Lachnospiraceae family but increase in Porphyromonadaceae family (phylum Bacteroidetes) (De Souza-Basqueira et al., 2020).

Overall, all the main gut bacteria phyla play a prominent role in colonization resistance, but some members may also be involved in inflammation. For example, *Lactobacillus* spp. from the Firmicutes phylum are able to inhibit *C. difficile* colonization and reduce inflammation, while segmented filamentous bacteria (SFB), also from the Firmicutes phylum, induce the secretion of antimicrobial peptides (AMPs), pro-inflammatory cytokines and IgA, as well as trigger the development of $CD4^+$ T helper 17 (T_H17) cells (Buffie and Pamer, 2013). Besides, studies now suggest that changes in the gut microbiota community may be associated with increased infection susceptibility (Libertucci and Young, 2019). However, since our understanding of the microbiota in CD is still in its infancy, studies are yet required to clarify the role of certain microorganisms in determining higher susceptibility to infection or even infection clearance, since many of them may present trypanolytic activity and therefore are vital for CD modulation.

Taken together, the aforementioned data show that *T. cruzi* infection is followed by changes in the gut microbiome and metabolome, which might be important for the parasite persistence in the host. Besides, the importance of other microbiota (oral and skin) to the pathogenesis of CD is also highlighted and deserves to be more defined in future studies. Nonetheless, these studies are preliminary and did not explore the *consequences* of these gut microbiota changes in the human population.

Vector–Parasite–Microbiota Interaction in Insects

Studies performed with triatomine vectors are also important to our current understanding of the role of the gut microbiota in CD. For example, a study was conducted to determine the microbiota changes that results from *T. cruzi* infection in *Rhodnius prolixus*, a vector of *T. cruzi* (Castro et al., 2012). In this study, *R. prolixus* were fed rabbit blood with or without the Dm28c clone of *T.cruzi*, which can complete its developmental cycle in the insect gut (Vieira et al., 2016). After insects were fed with blood, analyses of the gut microbiota using colony

forming unit (CFU) assay were performed from day 5 to 29, as well as antibacterial and phenoloxidase (PO) assays and NO measurement. Insects that were infected with *T. cruzi* presented low gut microbiota population as revealed by less CFU numbers in the agar plates (Vieira et al., 2016). Interestingly, a similar study using the same *T. cruzi strain* showed that after infection there were lower CFU counts and reduced numbers of *Serratia marcescens* (Enterobacteriaceae family), a bacterium with trypanolytic activity (Da Mota et al., 2019). Furthermore, reduced numbers of *Rhodococcus rhodnii* were also reported (Vieira et al., 2016). This bacterium belongs to Nocardiaceae family and helps in the processing of B vitamins in the triatomine's gut (Rodríguez et al., 2011; Pachebat et al., 2013).

Accordingly, another study showed that infection of six different species of *T. cruzi* vectors (*P. megistus*, *R. prolixus*, *T. brasiliensis*, *T. infestans*, *T. juazeirensis*, and *T. sherlocki*) with *T. cruzi epimastigotes* strain 0354 resulted in changes in gut microbiota community depending on the studied hosts, in a "species-specific manner," as well as in increased diversity in gut microbiota as demonstrated by 16s rRNA sequencing (Díaz et al., 2016). Furthermore, the gut antimicrobial activity was increased in *T.cruzi*-infected insects, as measured by inhibition zone and turbidometric assay (Castro et al., 2012). However, the antimicrobial activity did not result in the parasite's elimination and it is rather explained by reduction in the microbial population, leading to low microbiota population (Castro et al., 2012). Moreover, the activity of PO, an important enzyme of the innate immune system of insects, and NO, an immune system mediator, were also investigated (Castro et al., 2012). The authors found that, PO activity levels were elevated and NO was decreased in infected insects. However, the increase in PO did not correlate with reduction of parasite density in the gut (Castro et al., 2012). Although PO activity levels were not enough for killing the parasite, it may be linked with changes in gut microbiota composition observed in the vector insects. These studies highlights that (i) *T.cruzi* interacts with triatomine vector microbiome and causes changes in the microbiome; (ii) the microbiome seems to be important for control of *T. cruzi*, since antibiotic treated insects had higher parasite density in the gut (Castro et al., 2012); (iii) vectorial competence may be associated with microbiota profile of vector insects; (iv) the microbiota changes observed after *T.cruzi* infection alter vector immunity.

In sum, insects studies show that *T. cruzi* infection is associated with changes in the gut microbiota, especially reduction in bacterium with trypanolytic activity and increased diversity of gut microbiota. *T. cruzi* infection also increased the secretion of AMPs, leading to a reduction in the gut microbiota community, rather than eliminating the parasite.

MICROBIOTA, CD, AND ITS COMORBIDITIES

As already discussed, heart inflammation is a key feature of CD. Interestingly, data are now suggesting that microbial-derived molecules and metabolites, such as microbe-associated molecular patterns (MAMPS), SCFAs, 2BAs, and TMAO may underpin cardiovascular disease (CVD) (Howitt and Garrett, 2012; Zhang and Davies, 2016; Brown and Hazen, 2018). Interestingly, some gut microbes can produce trimethylamine (TMA), which results from the metabolism of fat-rich nutrients by enzymes known as microbial TMA lyases. After being produced, TMA can then enter the portal circulation and the liver, where it is further processed to generate TMAO, which triggers heart and kidney dysfunction, such as atherosclerosis, heart failure, renal fibrosis (Brown and Hazen, 2018). Furthermore, studies now acknowledge that CD patients frequently suffer from other comorbid conditions, such as major depressive disorder (MDD) and anxiety (Hueb and Loureiro, 2005; Marchi and Gurgel, 2011; Ozaki et al., 2011; Jackson et al., 2012; Suman et al., 2017). Unsurprisingly, the gut microbiome has been associated with the development of these neuropsychiatric disorders (Mayer et al., 2014; Sharon et al., 2016; Dinan and Cryan, 2017; Cryan et al., 2019). It should be noted that CD also affects the enteric nervous system (ENS) (Meneghelli, 1985; Iantorno et al., 2007). Unsurprisingly, the gut microbiome has been recognized to take part in the modulation of ENS development and function (Obata and Pachnis, 2016; Obata et al., 2020) and the ENS is able to modulate the gut microbiome (Rolig et al., 2017). Based on the data mentioned above, one could speculate on whether the gut microbial community would be an additional underpinning mechanism behind mood, cardiovascular, and ENS changes in CD. Nevertheless, this needs to be further investigated and corroborated.

EMERGING VIEW OF CD ON THE LIGHT OF THE MICROBIOTA: HOW DOES THE GUT MICROBIOTA CHANGE OUR CURRENT VIEW OF CD?

As discussed in the introduction section, CD was previously considered a disease resulting only from the parasite-host interaction, but currently this view is changing due to the inclusion of the host-microbiome as new partner in this intricate relationship. As a result, studies are now focusing on the "*vector–parasite–microbiota* interaction" (Díaz et al., 2016) and new insights on the pathogenesis of CD are consequently emerging. Unfortunately, the data collected so far does not precisely draw a new picture of CD, but they indeed point toward a new direction researchers should look at when designing new experiments. This fact is relevant because these new studies will probably lead to the discovery of new bacteria with essential activities for modulation of *T. cruzi* infection and infection resolution. At a later stage, these newly discovered bacteria may be manipulated either pharmacologically or genetically (Taracena et al., 2015) to benefit the host directly or indirectly. Identifying bacterial communities present in the human microbiome that can act against *T. cruzi* is a task that remains unresolved and therefore, should be urgently addressed.

Throughout this review, we could observe that *T. cruzi* infection results in alterations in the gut microbiota of triatomine vectors, and this allows the parasite to progress and establish

infection. Later, when these vectors are in contact with humans or animals and transmit the parasite to these new hosts, *T. cruzi* again induces changes in their gut microbiota. Here, we postulate that the same process that happens in insects occurs in humans or mice: the parasite induces changes in bacteria population. These changes are responsible for alterations in the fecal metabolome, impaired immune response, and poor resolution of disease, leading to its persistence in the host. If that is the case, then the parasite uses the same *modus operandi* for insects and human hosts. Nevertheless, this hypothesis still needs to be confirmed by future studies.

NEW PERSPECTIVES ON THE TREATMENT OF CD: FOCUS ON PREBIOTICS AND PROBIOTICS

Benznidazole and nifurtimox are the only available drugs used to treat CD over the last 40 years. They are especially effective in curing the disease when employed in the acute phase. However, their effectiveness declines when they are used during the chronic phase (Clayton, 2010). One of the main problems regarding CD treatment is the *timing* and *side effects* of current therapies (Clayton, 2010). For instance, a period of 60 days is required for benznidazole to cure the disease at the early stage and this drug usually has severe side effects (Clayton, 2010). This makes adhesion to treatment and prescription of these drugs a big issue that needs to be urgently tackled. Interestingly, benznidazole may improve CD in association with gut microbiota modulation, as infected children treated with this drug had decreased *Prevotella* and *Coprococcus* (phylum Firmicutes), increase in the amount of *Dialister* (phylum Firmicutes) and *Enterobacteriaceae* (phylum Proteobacteria) (Robello et al., 2019).

The fact that gut microbiota is somehow related to CD etiopathogenesis undoubtedly opens up the possibility of development and the use of new treatments for CD. In this regard, *prebiotics* – non-digestible fibers metabolized by the gut microbiota – and *probiotics* – living organisms that may promote health benefits – deserve special focus since they are already being tested preclinically for other diseases, such as irritable bowel syndrome (IBS) (Başturk et al., 2016; Chen et al., 2017; Ford et al., 2018; Trifan et al., 2019), although the results are still preliminary. Regarding CD and the use of these treatments, there is hitherto only one report using probiotic and none using

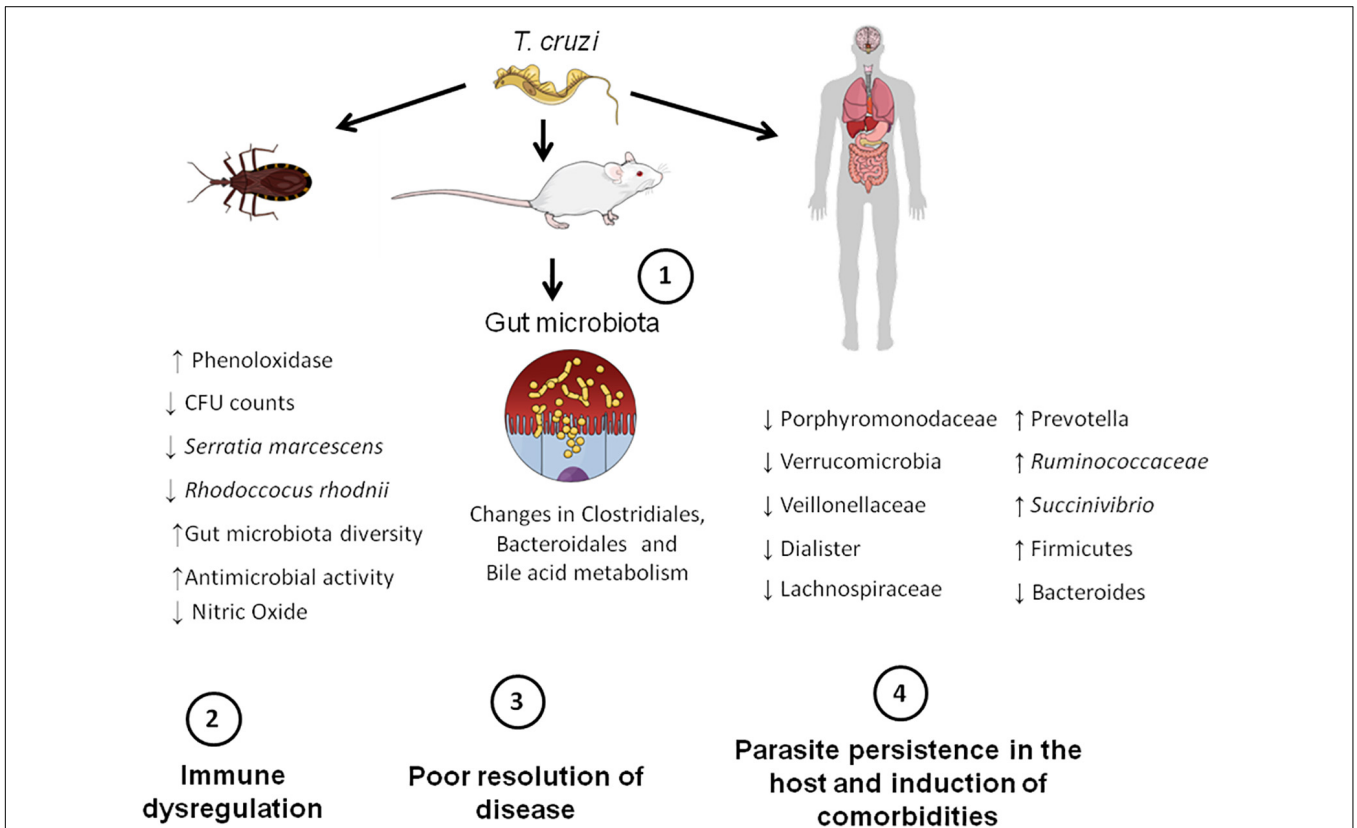

FIGURE 2 | Microbiota-host communication pathways in CD. Firstly, *T. cruzi* establishes infection in the mammalian (mice and human) and insect hosts (insect vectors). This initial step triggers gut microbiota changes in the aforementioned hosts. For example, in insects, infection leads to a decreased number of bacterial species that possess trypanolytic activity, such as *Serratia marcescens*, which increases the susceptibility of the host to *T. cruzi* infection. In mice, changes in the order Bacteroidales and Clostridiales, as well in the bile acid metabolism take place. In humans, there is an overall increase in Firmicutes phylum and decrease in Bacteroides. These gut microbiota changes induced by the parasite lead to immune dysregulation and consequently poor resolution of disease. Finally, the parasite persists in the host and is able to induce comorbidities, such as major depressive disorder (MDD).

prebiotics. Therefore, whether they may be a valid and efficacious treatment for CD will only be confirmed in the future. However, probiotics as therapeutic agents against parasitic diseases have long been proposed (Travers et al., 2011).

Gut microbiota-directed interventions hold promise for CD treatment since some genres of bacteria can modulate immunity, which is known to be impaired in CD patients. By its immunomodulatory effects the host may be able to mount a more potent immune response against *T. cruzi*, but whether this is effective in preventing or eliminating infection is still largely unknown and begs more research in the future. In fact, only one study showed positive effects of probiotics in experimental CD. *Lactobacillus casei* was administered either orally or intraperitoneally to Swiss female mice 7 days prior to infection with *T. cruzi* (Ninoa strain) and the authors observed decreased parasitemia in the groups that received probiotics (Garfias et al., 2008). This study raises many important issues that deserve to be addressed: (a) the authors used a preventive or prophylactic approach, in which probiotics were given before infection; however, to determine what effects probiotics have in infected subjects, studies also need to focus on the therapeutic approach, in which probiotics are given after infection; (b) probiotics administration led to reduced number of blood parasites, not to the complete elimination of parasites from the host and therefore they may not be suitable to be used as monotherapy in the treatment of CD. Probiotics may, however, be useful as co-therapies with standard drugs, but this still needs to be tested. Therefore, more studies with pre- and probiotics are required, especially because, once their effectiveness is proven experimentally and in the clinical setting, patients may benefit from the fact that these are low-cost and non-invasive therapies, with fewer side effects than the current drugs used to treat CD. In sum, an in-depth understanding of the gut microbiota in CD will allow us to develop alternative therapeutic approaches to target CD, such as gut microbiota-directed interventions.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Here we provide pre-clinical and clinical evidence that gut microbiota may play a role in CD pathogenesis once it interferes with infection and its resolution. It became evident that *T. cruzi* induces changes in the gut microbiota and, especially in insects and mice, this is associated with a deregulated immune response and changed fecal metabolome, which might explain why the parasite persists in the host (**Figure 2**). However, the amount of data hitherto available is minute, and therefore, more studies are required to support the role of gut microbiota dysfunction in CD and its comorbidities. Future studies should test the effects of prebiotics and probiotics on preclinical and clinical CD to provide new treatment options for CD. Besides, they should investigate in depth the role of gut microbiota in CD pathogenesis, focusing on the role of microbes of certain microbial groups, such as Firmicutes and Bacteroidetes, and its impact in *T. cruzi* infection. Exploring the effects of *T. cruzi* infection in humans and what microbiota changes are caused by the parasite, as well as what species of microbes could be modulated to control disease are also necessary. Addressing the effects of different *T. cruzi* strains on microbiota changes as well as to explore the role of skin and oral microbiota in CD is also encouraged. Interestingly, other issues, such as the microbiota-gut-brain axis are currently underexplored in CD and thus deserve future attention.

A question that deserves attention is: is the dysfunction in the microbiota responsible for neuropsychiatric comorbidities observed in CD patients and could it be prevented or delayed by the use of gut microbiota-directed interventions? Answering this would allow us not only to better tackle CD but also its comorbidities. Moreover, given that pre- and probiotics are molecules derived from dietary compounds, such as vegetables and fruits, it is also essential to test whether a diet rich in these molecules could modulate CD and ameliorate disease state in the patients.

Other gut microbiota-targeted therapies that might have relevance to treating CD are fecal microbiota transplantation (FMT) and *synbiotics*. Although appealing, no data is hitherto available to support their use in CD patients. Furthermore, as there are considerable differences in the microbial profile of different portions of the gut (Donaldson et al., 2015), future studies should also employ colon biopsies to profile the microbial community in the intestinal mucosa of patients with CD.

As a matter of fact, funds for drug Research and Development (R&D) on CD are scarce, which happens for many reasons (Clayton, 2010), which are beyond the scope of this paper. Employing the suggestions mentioned above would probably draw the government and pharmaceutical companies' attention to more funding for CD, probably leading to an increase in studies on the current topic and more elucidation on CD pathogenesis. As a consequence, CD would be less neglected than it is today.

AUTHOR CONTRIBUTIONS

ED-S conceived the study, performed the literature search, data collection, data analysis, wrote the manuscript, and created the figures under the supervision of CP. LM contributed to the writing of the manuscript. CP, GC, LM, and WS critically reviewed the manuscript. All authors approved the final version of this manuscript.

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GLOSSARY

Germ-free (GF) mice – mice raised without a microbiome.

Y strain of *T. cruzi* – A strain that is associated with low parasitemia, high mortality in mice and that can also infect neurons and glia.

Trypomastigotes – The flagellated form of *T.cruzi*. Trypomastigotes are released in the insect vector feces when the insect takes a blood meal. They enter the host via mucosa or wounded sites and then infects mammalial cells, loses the flagellum and becomes amastigotes, which replicate by binary fission. Next, they turn back to trypomastigotes, causing cell lysis and spread of infection

Polysaccharide A (PSA) – a polysaccharide from the capsule of *B. fragilis* that has immunomodulatory effects.

Gnotobiotic mice – mice with only certain known bacterial strains/mice colonized with known bacterial strains.

Epimastigotes – non-infective form of *T. cruzi* detected in the gut of insect vectors. They replicate by binary fission. At a later stage during its life cycle, they become trypomastigotes.

Pathobiont – microorganisms of the microbiota with the potential to induce pathology (Round and Mazmanian, 2009).

Segmented filamentous bacteria (SFB) – gram-positive, spore-forming bacteria that modulates the immune system and attach to intestinal epithelium.

Antimicrobial peptides (AMPs) – peptides from the innate immune system with activity against viruses, fungi, parasites and bacteria.

Short-chain fatty acids (SCFAs) – bacterial metabolites, such as acetate, butyrate and propionate, derived from the fermentation of complex indigestible fibers.

Trimethylamine (TMA) – molecule that results from the metabolism of fat-rich or TMA-rich nutrients.

Trimethylamine-*N*-oxide (TMAO) – a microbial metabolite derived from metabolism of trimethylamine (TMA).

Enteric nervous system (ENS) – a network formed by neurons and glia in the gastrointestinal tract.

Prebiotics – non-digestible complex fibers that are fermented by the gut bacteria and promote the growth of some bacteria and health benefits.

Probiotics – consists of living microorganisms that confer health benefits when consumed in sufficient amounts.

Microbiota-gut-brain axis – the network composed of neural, immune and endocrine systems that mediate the effects of the gut microbiota in the host.

Fecal Microbiota Transplantation (FMT) – one type of microbiota directed intervention that consists in transferring faucal material from the donor to the recipient.

Secondary Bile Acids – molecules resulting from the microbes-driven metabolism of primary bile acids.

Synbiotics – association of prebiotics with probiotics.

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Probiotic *Lactobacillus rhamnosus* GG Promotes Mouse Gut Microbiota Diversity and T Cell Differentiation

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Lactic acid bacteria (LAB) are the primary genera of the intestinal flora and have many probiotic functions. In the present study, *Lactobacillus rhamnosus GG* (LGG) ATCC 53103 was used to treat BALB/c mice. After LGG intervention, both low and high LGG doses were shown to improve the observed OTU, Chao1, ACE, and Shannon indices, while the Simpson index decreased, demonstrating that LGG can promote intestinal microbiota abundance and diversity. Furthermore, LGG treatment increased the abundances of intestinal *Firmicutes*, *Bacteroides* and *Actinomycetes* while reducing that of *Proteobacteria*. In addition to its effect on gut the microbiota, LGG could also regulate the host immune system. In the present study, we showed that LGG could affect the percentage of $CD3^+$ T lymphocytes in the spleens (SPLs), mesenteric lymph nodes (MLNs), Peyer's patches (PPs) and lamina propria lymphocytes (LPLs) of mice, including total $CD3^+$ T, $CD3^+CD4^+$ T, and $CD3^+CD8^+$ T lymphocytes. Furthermore, LGG could effectively increase the expression of Th1-type cytokines (IFN-γ) and Th2 cytokines (IL-4) in $CD4^+$ T cells, indicating that the proportion of Th1 and Th2 cells in mice with LGG treatment was in a high equilibrium state compared to the control group. In addition, the IFN-γ/IL-4 ratio was greater than 1 in mice with LGG intervention, suggesting that LGG tends to mediate the Th1 immune response. The results of the present study also showed that LGG upregulated the expression of IL-17 in $CD4^+$ T cells and regulated the percentage of $CD4^+CD25^+Foxp3^+$ Treg cells in various secondary immunological organs, indicating that LGG may promote the balance of Th-17 and Treg cells.

Keywords: *Lactobacillus rhamnosus*, gut microbiota, T lymphocytes, probiotic, mucosal immunity

INTRODUCTION

Lactobacillus is the primary genus of the intestinal flora and probiotics of animals, and it is able to ameliorate the imbalance of the intestinal flora and aid in maintaining a number of biological functions of the immune system (Owyang and Wu, 2014). Studies indicated that *Lactobacillus rhamnosus* LS-8 and *Lactobacillus crustorum* MN047 supplementation possessed the anti-obesity

effect on the HFFD fed mice by alleviating inflammatory response and regulating gut microbiota, which further suggested that these two probiotics can be considered as an alternative dietary supplement in combination with the preventive and therapeutic strategies against obesity and related complications (Wang et al., 2020).

Among lactic acid bacteria (LAB), *Lactobacillus rhamnosus* GG (LGG)is the most frequently used strain. LGG strain is a Gram-positive bacteria isolated from the human digestive system for the first time in 1985 by Sherwood Gorbach and Barry Goldin. LGG regulate the intestinal flora through the mechanism of "competitive rejection," where the colonization of pathogens in the intestinal mucosa is hindered by increasing competition for adhesion sites and nutrients, production of antibacterial compounds (Petrova et al., 2016; Tytgat et al., 2016), regulation of gut microbiota homeostasis (Berni Canani et al., 2016), maintaining function of the intestinal barrier (Zhang et al., 2015), as well as modulation of local or systemic immune response (Johansson et al., 2016). Previous study showed LGG administration enables reprogramming of microbe-microbe interactions and alters ileal microbiota with associated specific $CD3^{-}CD19^{-}$cell subset homeostasis. LGG reduces the cell population in the Prevotellaceae NK3B31 group, changes the correlation network in Prevotellaceae NK3B31 group-centric species, and promotes symbiotic synergism of Fusobacterium, Lactobacillus animalis, and Propionibacterium (Zhang et al., 2019). LGG granules administration could decrease the number of *Clostridium perfringens* and increase the *Lactobacillus* and *Bifidobacterium* in the intestine of alcohol-induced mice, which suggested LGG granules prevent alcohol-induced intestinal flora disorder, increase Gram-positive bacteria, decrease Gram-negative bacteria that induce LPS accumulation, and reduce fat accumulation and inflammatory response in liver, so as to ameliorate the liver damage (Gu et al., 2020). LGG supplementation mainly increased the *Bacteroidetes* population in the nude mice. The high abundance of *Bacteroidetes* and *Alistipes* resulted in a high butyrate level in the nude mice treated with LGG to promote butyrate production, protecting against deoxynivalenol exposure in nude mice (Lin et al., 2020).

Lactobacillus can regulate both innate and adaptive immunity, playing a role in disease prevention and treatment in the host through immune stimulation and regulation. The effect of *Lactobacillus* on innate immunity is primarily achieved by enhancing the phagocytic ability of the monocyte-phagocytic system. Studies have shown that *Lactobacillus* can enhance the phagocytic activity of mouse peritoneal macrophages and play a regulatory role in the immune system (Donnet-Hughes et al., 1999). In addition, neonatal treatment of p40, a LGG-derived protein, reduced the susceptibility to intestinal injury and colitis and promoted protective immune responses in adult mice, including IgA production and differentiation of regulatory T cells. These findings reveal novel roles of neonatal supplementation of probiotics-derived factors in promoting EGFR-mediated maturation of intestinal functions and innate immunity, which likely promote long-term beneficial outcomes. Furthermore, p40 promotes IgA production through upregulation of APRIL expression in intestinal epithelial cells and mucin production through activation of epidermal growth factor receptor (Shen et al., 2018).

Our recent study also found that LGG can protect the development and integrity of intestinal villi, maintain the integrity of intestinal villi, and promote the growth of villi length. LGG can also regulate the proliferation of T-lymphocytes in the intestine of early weaning piglets at 30 days and 45 days, and increase the number of CD3+ CD4+ T-lymphocytes (Shonyela et al., 2020). Colonization of germ-free mice with either *Lactobacillus johnsonii* (NCC 533) or *Lactobacillus paracasei* (NCC 2461) induced similar germinal center formation and immunoglobulin A-bearing lymphocytes in the mucosa, suggesting that LAB can activate mucosal B-cell responses (Ibnou-Zekri et al., 2003). Our results found that LGG intervention can promote the development and maturation of B lymphocytes, enhance the activation and antigen-presentation ability of B lymphocytes, and regulate the secretion of immunoglobulin by B lymphocytes. Thus, LGG can regulate the mucosal immunity and humoral immunity of mice (Shi et al., 2020).

In addition, the relationship between modulation of gut microbiota and regulation of host immunity by different doses of probiotics is complicated. LGG exerted divergent dose-dependent effects on the intestinal immune cell signaling pathway responses, with 9-doses LGG being more effective in activating the innate TLR9 signaling pathway than 14-doses in the HGM pigs vaccinated with AttHRV (Wang et al., 2020). Thus, in the present study, by using different doses of LGG to intervene in mice, we found that the changes of intestinal flora caused by different doses of lactic acid bacteria had different effects on the proliferation and differentiation of T lymphocyte subsets in mice. The application measurement of lactic acid bacteria is still to be discussed.

RESULTS

Alpha Diversity of the Gut Microbiota

After quality trimming and chimera checking, 576,311 high-quality 16S rRNA gene reads were obtained from the 15 samples that had an average length of 437.10 bp. The Good's coverage index was greater than 99%, and 6390 OTUs were identified from all samples. Rarefaction, Shannon, Coverage and OTU rank-abundance curve analysis were used to standardize and compare the observed taxon richness among samples and to determine whether the contents of all samples were unequally sampled. The results suggested that the sequencing depth was sufficient to cover the microbial diversity of each sample and that additional data would not yield more OTUs (**Supplementary Figure 1**).

In the present study, the Chao1, ACE, Simpson, and Shannon indices were used to evaluate the richness and alpha diversity of the gut microbiota (**Figure 1**). The Chao1 and Ace indices can be used to evaluate the richness of the intestinal microbiota, which is proportional to the richness of the gut microbiota, while the Simpson and Shannon indices can be used to evaluate the diversity of the gut microbiota, higher values of which indicate lower and greater microbiota diversity, respectively. The results

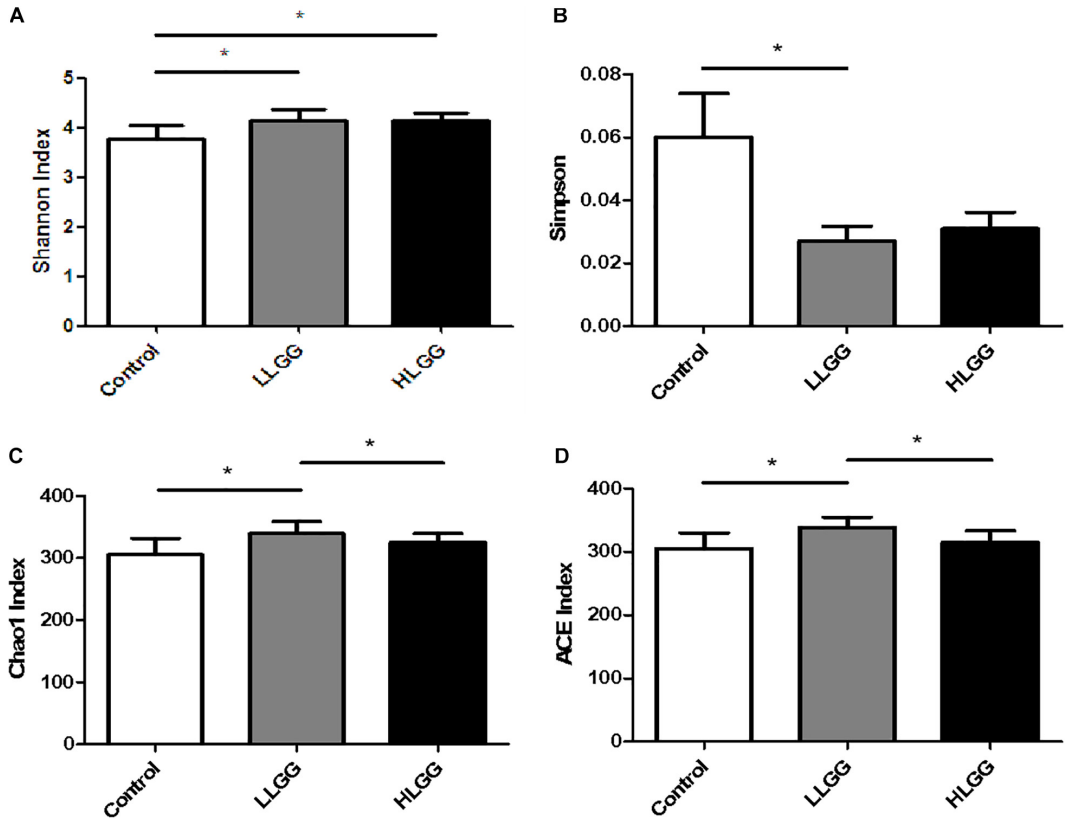

FIGURE 1 | Analysis of different microbial alpha diversity indices in the three experimental groups. The Shannon **(A)** and Sipson **(B)** indices were used as diversity estimators. The Chao1 **(C)** and ACE **(D)** indices were used as richness estimators. *Indicated significant differences ($p < 0.05$) by students' *t*-test.

showed that after 7 LGG interventions, compared to the control group, the Chao1, ACE, and Shannon indices in the LLGG group significantly increased, whereas the Simpson index significantly decreased, indicating that the low-dose LGG treatment could promote the richness and diversity of the gut microbiota. Compared to the control group, the Shannon index significantly increased in the HLGG group, while no significant differences were observed between the Chao1, ACE, and Simpson indices, indicating that the high-dose LGG treatment could promote gut microbiota diversity. Compared to the HLGG group, the Chao1 and ACE indices in the LLGG group significantly increased, while the Shannon and Simpson indices showed no significant changes. Thus, based on the observed Chao1, ACE, Simpson, and Shannon indices, LGG could promote the alpha diversity of gut microbiota, and the low-dose LGG treatment could better promote the richness and diversity of the gut microflora.

LGG Pretreatment Affects the Gut Microbiota Structure

Venn diagrams were generated to evaluate the distribution of OTUs among the different treatment groups. As shown in **Figure 2A**, the taxon-independent Venn analysis results showed that 333 OTUs were shared among the three groups, with the total number of OTUs being highest in the HLGG group, followed by the LLGG and control groups. The unique number of OTUs was 9, 12, and 6 in the control, LLGG and HLGG groups, respectively. The predominant genera co-occurred among the three groups, while the abundances of some rare genera differed. The genera *Butyricicoccus*, Ruminiclostridium_5, *[Eubacterium]_brachy_group* and *unclassified_f__Erysipelotrichaceae* were unique to the control group. The genera *norank_o__Mollicutes_RF9* were unique to the LLGG group. The genera *norank_o__Gastranaerophilales*, *Alloprevotella* and *[Eubacterium]_coprostanoligenes_group* were unique to the HLGG group (**Figure 2B**) To elucidate the effect of LGG on the composition and structure of the gut microflora, we assessed the community composition of each sample at the phylum and genus levels. The sequencing results showed the presence of 10 phyla, of which *Firmicutes* and *Bacteroidetes* were the primary phyla in all three treatment groups. In addition, *Verrucomicrobia*, *Deferribacteres,* and *Proteobacteria* were present in all samples. In the control group, the relative abundances of *Bacteroidetes*, *Firmicutes*, *Verrucomicrobia*, *Proteobacteria,* and *Deferribacteres* were 39.00, 38.85, 9.51, 9.91, and 1.16%, respectively. In the LLGG group, the relative abundances of *Bacteroidetes* and *Firmicutes* increased to 45.12 and 39.89%, while those of *Verrucomicrobia*, *Proteobacteria,* and *Deferribacteres* decreased to 8.05, 4.24, and 1.62%, respectively. In the HLGG group, the relative

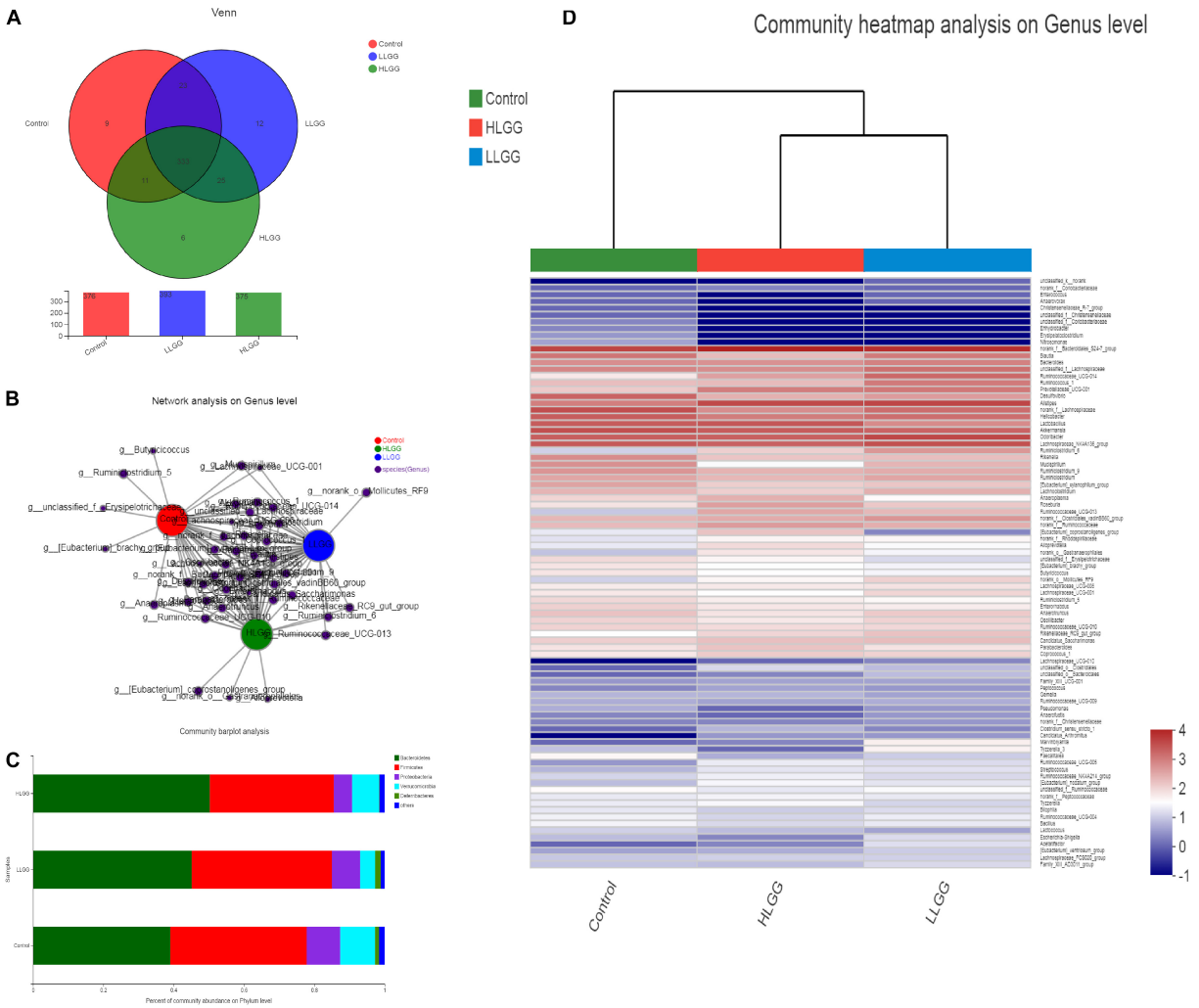

FIGURE 2 | Changes in gut microbiota communities of BALB/c mice in response to LGG pretreatment. **(A)** Venn diagram of common and unique OTUs among the three groups. The numbers of observed OTUs sharing ≥97% nucleotide sequence identity. **(B)** A network diagram showing the OTUs among the three groups. **(C)** Gut microbiota communities at the phylum level. The stacked bars show the combined relative abundances of phylum-level taxa per animal. Colors are assigned for all phyla detected. **(D)** Heatmap showing the spatial distributions of all OTUs at the genus level.

abundance of *Bacteroidetes* increased to 50.26%, while the abundances of *Firmicutes*, *Verrucomicrobia* and *Proteobacteria* and *Deferribacteres* decreased to 35.30, 5.23, 7.64, and 0.16%, respectively (**Figure 2C**). The heatmap-based analysis at genus level showed that the gut microbiota profiles of mice in the LLGG group were similar to those in the HLGG group (**Figure 2D**).

The PLS-DA results based on the relative abundances of OTUs clearly showed distinguishable ileal mucosal microbiota samples among the three groups (**Supplementary Figure 2**). To identify the specific phylotypes that were significantly altered in response to LGG supplementation, all effective sequences from the sample were analyzed using the LEfSe method When comparing the control and LLGG groups (**Figures 3A,B**), the relative abundance of *Proteobacteria* was enriched at the phylum level, while the relative abundances of *unclassified_f__Erysipelotrichaceae* and *Desulfovibrio* were enriched at the genus level in the control group. In contrast, in the LLGG group, the relative abundance of *Bacteroides* was enriched at the phylum level, and the relative abundances of *Marvinbryantia*, *Ruminococcaceae_UCG_014*, *Ruminiclostridium_6*, *Ruminococcaceae_UCG_013*, *norank_f__Clostridiales_vadinBB60_group*, *Prevotellaceae_UCG_001*, *norank_f__Bacteroidales_S24_7_group*, *Rikenellaceae_RC9_gut_group*, *Alistipes*, and *norank_o__Mollicutes_RF9* were enriched at the genus level. When comparing the control and HLGG groups (**Figures 3C,D**), the relative abundance of *Bacteroidetes* was enriched at the phylum level, while the relative abundances of *Roseburia*, *Eubacterium__nodatum_group*, *Prevotellaceae_UCG_001*, *Parabacteroides*, *norank_f__Bacteroidales_S24_7_group*, and *Alistipes* were enriched at the genus level in the HLGG group. In contrast, in the control group, the relative abundances of *Proteobacteria* and *Deferribacteres* were enriched at the

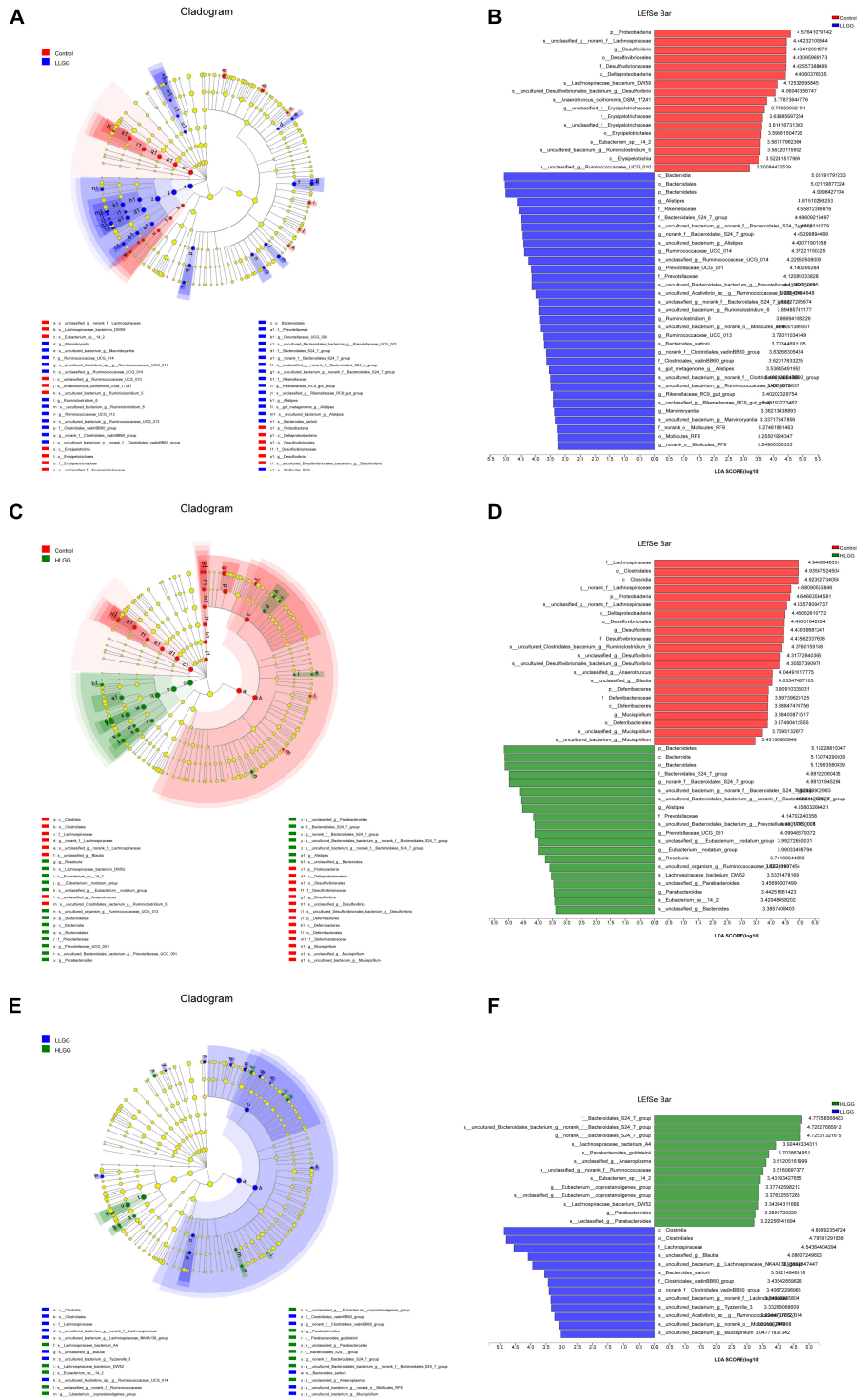

FIGURE 3 | LEfSe analysis results. Only the taxa with LDA scores higher than 3 are shown; **(A,B)** Cladogram showing the phylogenetic relationships of bacterial taxa and LDA scores between the control and LLGG groups. **(C,D)** Cladogram showing the phylogenetic relationships of bacterial taxa and LDA scores between the control and HLGG groups. **(E,F)** Cladogram showing the phylogenetic relationships of bacterial taxa and LDA scores between the LLGG and HLGG groups.

phylum level, while those of *norank_f__Lachnospiraceae*, *Desulfovibrio*, and *Mucispirillum* were enriched at the genus level. In addition, when comparing the LLGG and HLGG groups (**Figures 3E,F**), the relative abundance of *norank_f__Clostridiales_vadinBB60_group* was enriched at the genus level in the LLGG group, while the relative

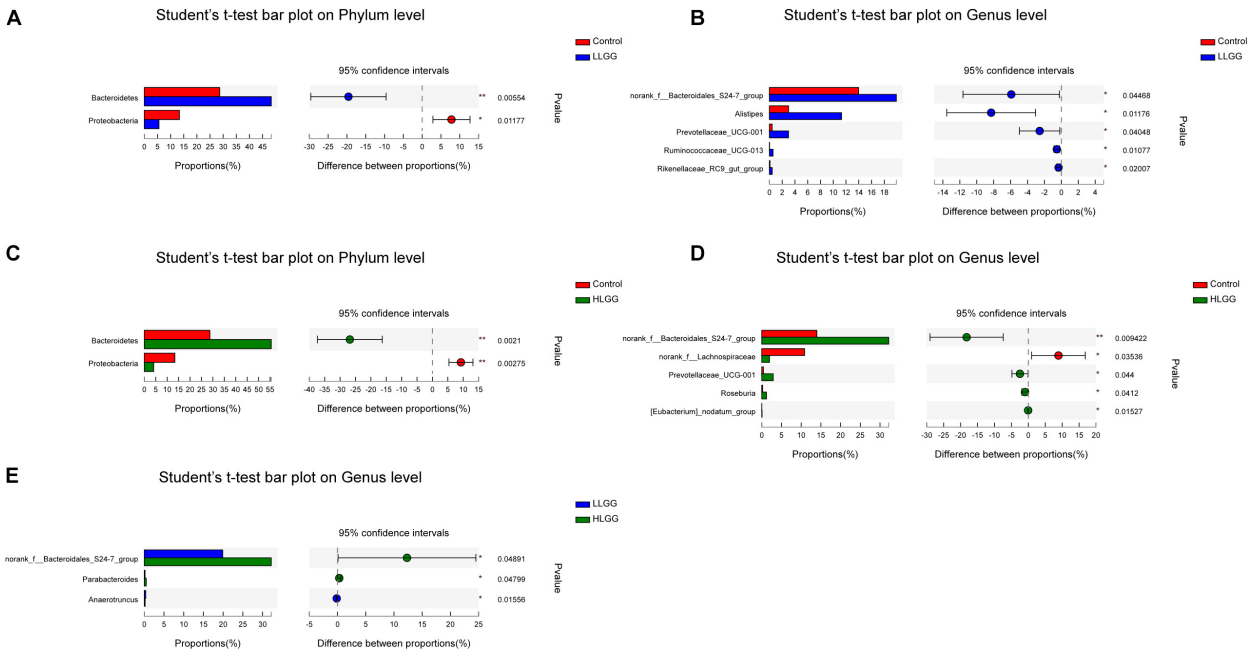

FIGURE 4 | Differences in the relative abundances of OTUs from the three indicated treatment groups were analyzed using Student's *t*-test. **(A–E)** The taxonomy of the OTUs (phylum and genus levels) is depicted on the left. The *Y*-axis shows the species name at a specific taxonomic level, and the *X*-axis shows the average relative abundance in different groups. The columns with different colors represent different groups, and the rightmost value is the *P*-value, *$0.01 < P \leq 0.05$, **$0.001 < P \leq 0.01$.

abundances of *Eubacterium__coprostanoligenes_group* and *norank_f__Bacteroidales_S24_7_group* were enriched in the LHGG group. Subsequently, Student's *t*-test was used to evaluate the LEfSe results. Compared to the control group, the relative abundance of *Bacteroides* significantly increased in the LLGG group, whereas that of *Proteobacteria* significantly decreased at the phylum level (**Figure 4A**), and the relative abundances of *norank_f__Bacteroidales_S24-7_group*, *Alistipes*, *Prevotellaceae_UCG-001*, *Ruminococcaceae_UCG-013,* and *Rikenellaceae_RC9_gut_group* significantly increased at the genus level (**Figure 4B**). Compared to the control group, the relative abundance of *Bacteroides* significantly increased in the HLGG group, while that of *Proteobacteria* significantly decreased at the phylum level (**Figure 4C**), and at the genus level, the relative abundances of *norank_f__Bacteroidales_S24-7_group*, *Prevotellaceae_UCG-001*, *Roseburia,* and *[Eubacterium]_nodatum_group* significantly increased, and that of *norank_f__Lachnospiraceae* significantly decreased (**Figure 4D**). Compared to the LLGG group, no significant difference was observed in the HLGG group at the phylum level, whereas at the genus level, the relative abundances of *norank_f__Bacteroidales_S24-7_group* and *Parabacteroides* were significantly increased and that of *Anaerotruncus* was significantly decreased (**Figure 4E**).

In addition, to characterize the functional changes of intestinal microbiome under LGG intervention, we predicted the functional composition profiles from 16S rRNA sequencing data with PICRUSt in HLGG, LLGG, and controls. Based on three groups of mice intestinal flora KEGG pathway (level 3) abundance of analysis, we found enrichment of intestinal flora pathways in LLGG group, including Biosynthesis of amino acids, Carbon metabolism, ABC transporters, and Ribosome pathways, etc (**Supplementary Figure 3**).

Effect of LGG on the Proportion of T Cells

CD3 participates in signal transduction after antigen recognition by TCR, and all T cells express CD3 molecules. After 7 LGG interventions, the percentages of $CD3^+$ T cells in the spleens (SPLs) of mice in the control, LLGG and HLGG groups were 32.9 ± 0.68%, 38.52 ± 0.88%, and 37.86 ± 0.88%, respectively. Compared to the percentage of $CD3^+$ T cells in the SPLs of mice in the control group, a higher percentage was observed in those of LLGG group ($p < 0.05$, $p = 0.001$) and HLGG group ($p < 0.05$, $p = 0.0007$). The percentages of $CD3^+$ T cells in the mesenteric lymph nodes (MLNs) of mice in the control, LLGG and HLGG groups were 74.84 ± 0.71%, 71.72 ± 0.27%, and 77.48 ± 0.63%, respectively. The percentage of $CD3^+$ T cells in the MLNs was lower in the LLGG group ($p < 0.05$, $p = 0.0035$) and higher in the HLGG group ($p < 0.05$, $p = 0.0246$) than that observed in the control group. The percentages of $CD3^+$ T cells in lamina propria lymphocytes (LPLs) in the control, LLGG and HLGG groups were 29.04 ± 0.89%, 32.30 ± 0.38%, and 33.38 ± 0.82%, respectively. Compared to the percentage of $CD3^+$ T cells observed in the LPLs of mice from the control group, higher percentages were observed in the LLGG group ($p < 0.05$, $p = 0.0101$) and HLGG group ($p < 0.05$, $p = 0.0072$).

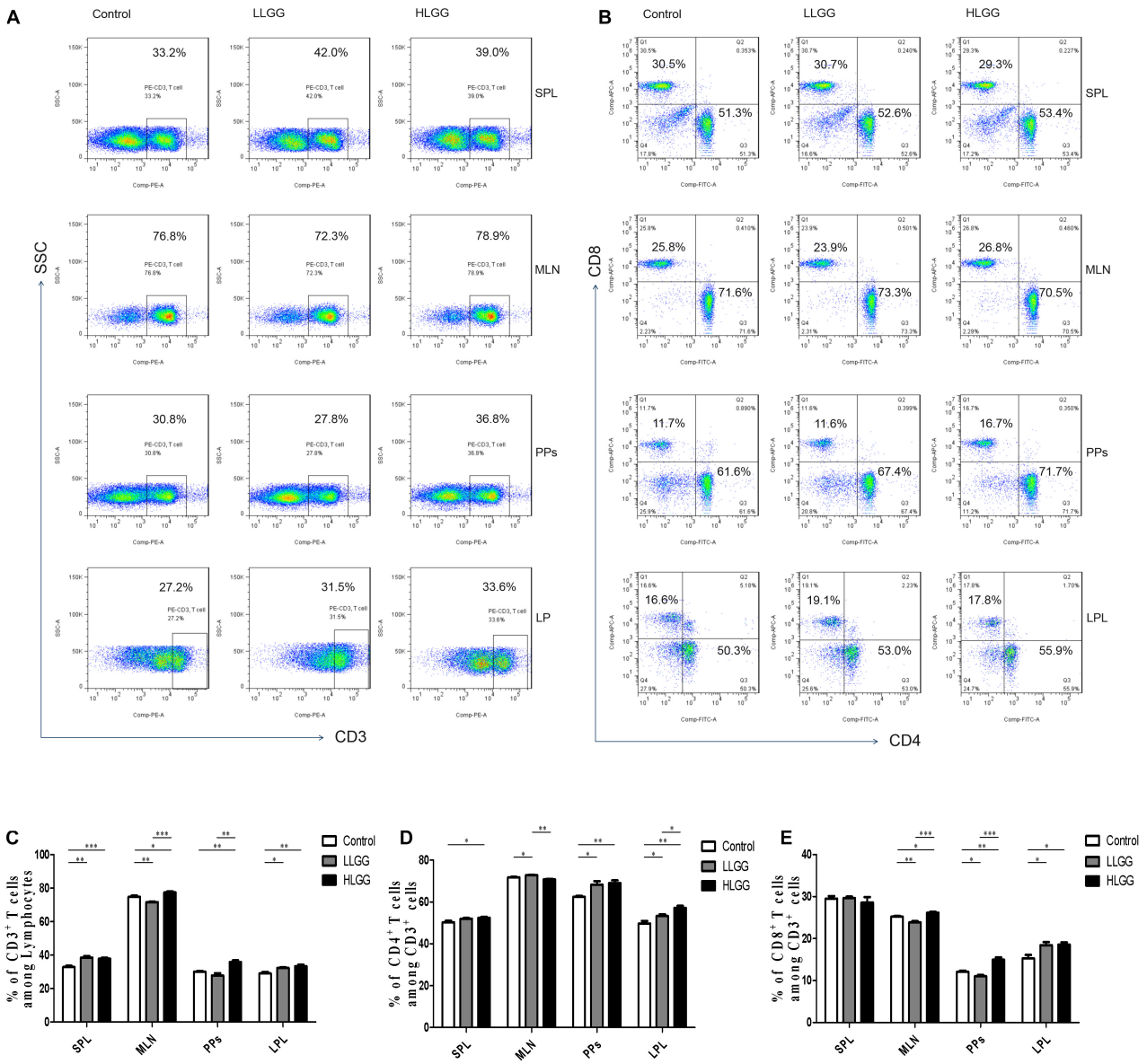

FIGURE 5 | LGG treatment increased the percentage of $CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ T cells in various secondary immunological organs. **(A)** Changes in the proportion of $CD3^+$ T cells in the SPLs, MLNs, PPs, and LPLs of mice treated with different doses of LGG, as shown by representative flow cytometry data. **(B)** Changes in the proportion of $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells in the SPLs, MLNs, PPs, and LPLs of mice treated with different LGG doses, as shown by representative flow cytometry data. **(C)** Statistical data showing the percentage of $CD3^+$ T cells in various secondary immunological organs from **(A)**. **(D,E)** Statistical data showing the percentage of $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells in various secondary immunological organs from **(B)**. *$0.01 < P \leq 0.05$, **$0.001 < P \leq 0.01$, ***$P \leq 0.001$.

The percentages of $CD3^+$ T cells in Peyer's patches (PPs) of mice in the control, LLGG and HLGG groups were 30.04 ± 0.50%, 27.82 ± 1.42%, and 35.86 ± 1.41%, respectively. The percentage of $CD3^+$ T cells in PPs from mice in the HLGG group was higher than that observed in the control group ($p < 0.05$, $p = 0.001$) and LLGG group ($p < 0.05$, $p = 0.0018$) (**Figures 5A,C**).

CD4 only exists in T cells that can recognize exogenous antigens presented by MHC-II, and $CD3^+CD4^+$ T cells are important immune cells. After 7 LGG interventions, the percentages of $CD3^+$ $CD4^+$ T cells among $CD3^+$ T lymphocytes in the SPL in the control and HLGG groups were 50.30 ± 0.72% and 52.48 ± 0.41%, respectively. The percentage of $CD3^+$ $CD4^+$ T cells among $CD3^+$ T lymphocytes in the SPLs of mice in the HLGG group was higher than that observed in the control group ($p < 0.05$, $p = 0.0310$). The percentages of $CD3^+$ $CD4^+$ T cells among $CD3^+$ T lymphocytes in MLNs from mice in the control, LLGG and HLGG groups were 71.76 ± 0.24%, 72.62 ± 0.27% and 70.96 ± 0.25%, respectively. The percentages of $CD3^+$ $CD4^+$

T cells among $CD3^+$ T lymphocytes in PPs from the control, LLGG and HLGG groups were 62.48 ± 0.46%, 68.30 ± 1.60% and 69.08 ± 1.29%, respectively. Compared to the percentage of $CD3^+$ $CD4^+$ T cells observed among $CD3^+$ T lymphocytes in PPs in the control group, higher percentages were observed in the LLGG group ($p < 0.05$, $p = 0.0101$) and HLGG group ($p < 0.05$, $p = 0.0014$). The percentages of $CD3^+$ $CD4^+$ T cells among $CD3^+$ T lymphocytes in the LPLs from the control, LLGG and HLGG groups were 49.76 ± 1.23%, 53.30 ± 0.84% and 57.20 ± 0.94%, respectively. The percentage of $CD3^+$ $CD4^+$ T cells among $CD3^+$ T lymphocytes in the LPLs from the HLGG group was higher than that observed in the control group ($p < 0.05$, $p = 0.0014$) and LLGG group ($p < 0.05$, $p = 0.0018$), while the percentage observed in the LLGG group was also higher than that measured in the control group ($p < 0.05$, $p = 0.0452$) (**Figures 5B,D**).

CD8 exists only in T cells that can recognize endogenous antigens presented by MHC-I and can kill abnormal cells, namely, cytotoxic T cells. After 7 LGG interventions, the percentages of $CD3^+$ $CD8^+$ T cells among $CD3^+$ T lymphocytes in SPLs from the control, LLGG and HLGG groups was not significantly different. The percentages of $CD3^+$ $CD8^+$ T cells among $CD3^+$ T lymphocytes in the MLNs from the control, LLGG and HLGG groups were 25.22 ± 0.17%, 23.86 ± 0.28%, and 26.16 ± 0.23%, respectively. The percentage of $CD3^+$ $CD8^+$ T cells among $CD3^+$ T lymphocytes in MLNs from the HLGG group was higher than that observed in the control group ($p < 0.05$, $p = 0.0129$) and LLGG group ($p < 0.05$, $p = 0.0003$), while the percentage in the LLGG group was lower than that observed in the control group ($p < 0.05$, $p = 0.0037$). The percentages of $CD3^+$ $CD8^+$ T cells among $CD3^+$ T lymphocytes in PPs from the control, LLGG and HLGG groups were 12.08 ± 0.26%, 11.01 ± 0.30%, and 14.96 ± 0.57%, respectively. The percentage of $CD3^+$ $CD8^+$ T cells among $CD3^+$ T lymphocytes in PPs from the HLGG group was higher than that observed in the control group ($p < 0.05$, $p = 0.0018$) and LLGG group ($p < 0.05$, $p = 0.0003$), while the percentage in the LLGG group was lower than that observed in the control group ($p < 0.05$, $p = 0.0291$). The percentages of $CD3^+$ $CD8^+$ T cells among $CD3^+$ T lymphocytes in the LPLs from the control, LLGG and HLGG groups were 15.28 ± 0.83%, 18.42 ± 0.73%, and 18.56 ± 0.52%, respectively. Compared to the percentage of $CD3^+$ $CD8^+$ T cells observed among $CD3^+$ T lymphocytes in the LPLs from the control group, a higher percentage was observed in the LLGG group ($p < 0.05$, $p = 0.0225$) and HLGG group ($p < 0.05$, $p = 0.0105$) (**Figures 5B,E**).

In summary, both low-dose and high-dose LGG intervention significantly affected the percentage of $CD3^+$ T, $CD3^+$ $CD4^+$ T and $CD3^+$ $CD8^+$ T cells in peripheral lymphoid organs (SPLs), intestinal secondary lymphoid organs (MLNs, PPs) and intestinal tissue (LP). In the LLGG group, a significant increase the percentage of $CD3^+$ T cells in the SPLs, $CD3^+$ $CD4^+$ T cells in the MLNs and PPs, and $CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ T cells in the LPLs was observed, while a significant decrease in the percentage of $CD3^+$ T and $CD3^+$ $CD8^+$ T cells was observed in the MLNs and PPs. Thus, a high dose of LGG had a strong effect on the percentage of all subsets of T cells, which can better improve the immune function of mice.

Effects of LGG on Th1 Cell Differentiation in Mice

BALB/c mice at 6 weeks of age received 7 high- or low-dose LGG treatments on alternate days, and a PBS control group was also established. SPLs, MLNs, PPs, and LP of the three groups of mice were isolated after the interventions to prepare single-cell suspensions. PMA, ionomycin, and BFA were added to the single-cell suspensions. Lymphocytes were collected 6 h later, and IFN-γ was labeled with a specific fluorescent antibody and detected by flow cytometry. IFN-γ, also known as type II interferon, is one of the most important types of Th1 cytokines.

After 7 LGG interventions, the percentages of $CD3^+CD4^+$ IFN-γ^+ Th1 cells in the SPLs in the control, LLGG and HLGG groups were 2.14 ± 0.08%, 3.142 ± 0.20%, and 3.054 ± 0.14%, respectively. Compared to the percentage of $CD3^+CD4^+$ IFN-γ^+ Th1 cells in the SPLs from the control group, a higher percentage was observed in the LLGG group ($p < 0.05$, $p = 0.0020$) and HLGG group ($p < 0.05$, $p = 0.0005$) (**Figures 6A,C**). The percentages of $CD3^+CD4^+$ IFN-γ^+ Th1 cells in the MLNs from the control, LLGG and HLGG groups were 2.080 ± 0.19%, 2.760 ± 0.12%, and 3.236 ± 0.14%, respectively. Compared to the percentage of $CD3^+CD4^+$ IFN-γ^+ Th1 cells in the MLNs from the control group, a higher percentage was observed in the LLGG group ($p < 0.05$, $p = 0.0166$) and HLGG group ($p < 0.05$, $p = 0.0012$). The percentage of $CD3^+CD4^+$ IFN-γ^+ Th1 cells in the MLNs from the HLGG group was also higher than that observed in the LLGG group ($p < 0.05$, $p = 0.0351$) (**Figures 6B,C**). Compared to the control group, the LLGG and HLGG groups both exhibited increased percentages of Th1 cells. In addition, the percentage of Th1 cells in the MLNs from the HLGG group was higher than that observed in the LLGG group.

After 7 LGG interventions, serum IFN-γ concentrations were detected by ELISA. Compared to the concentration of serum IFN-γ observed in the control group, a higher concentration was observed in the LLGG group ($p < 0.05$, $p = 0.0091$) and HLGG group ($p < 0.05$, $p = 0.0021$). The concentration of serum IFN-γ in the HLGG group was also higher than that observed in the LLGG group ($p < 0.05$, $p = 0.0073$) (**Figure 6D**).

Effects of LGG on Th2 Cell Differentiation in Mice

IL-4 molecules are primarily secreted by Th2 cells and can promote B cells to proliferate and induce $CD4^+$ T cells to differentiate into Th2 cells. After 7 LGG interventions, the percentages of $CD3^+CD4^+$IL-4^+ Th2 cells in the SPL in the control, LLGG and HLGG groups were 2.142 ± 0.0955%, 2.730 ± 0.218%, and 2.660 ± 0.14%, respectively. Compared to the percentage of $CD3^+CD4^+$IL-4^+ Th2 cells observed in the SPLs from the control group, a higher percentage was observed in the LLGG group ($p < 0.05$, $p = 0.0384$) and HLGG group ($p < 0.05$, $p = 0.0155$) (**Figures 7A,C**). The percentages of $CD3^+CD4^+$IL-4^+ Th2 cells in the MLNs from the control, LLGG and HLGG groups were 1.952 ± 0.147%, 2.428 ± 0.077%,

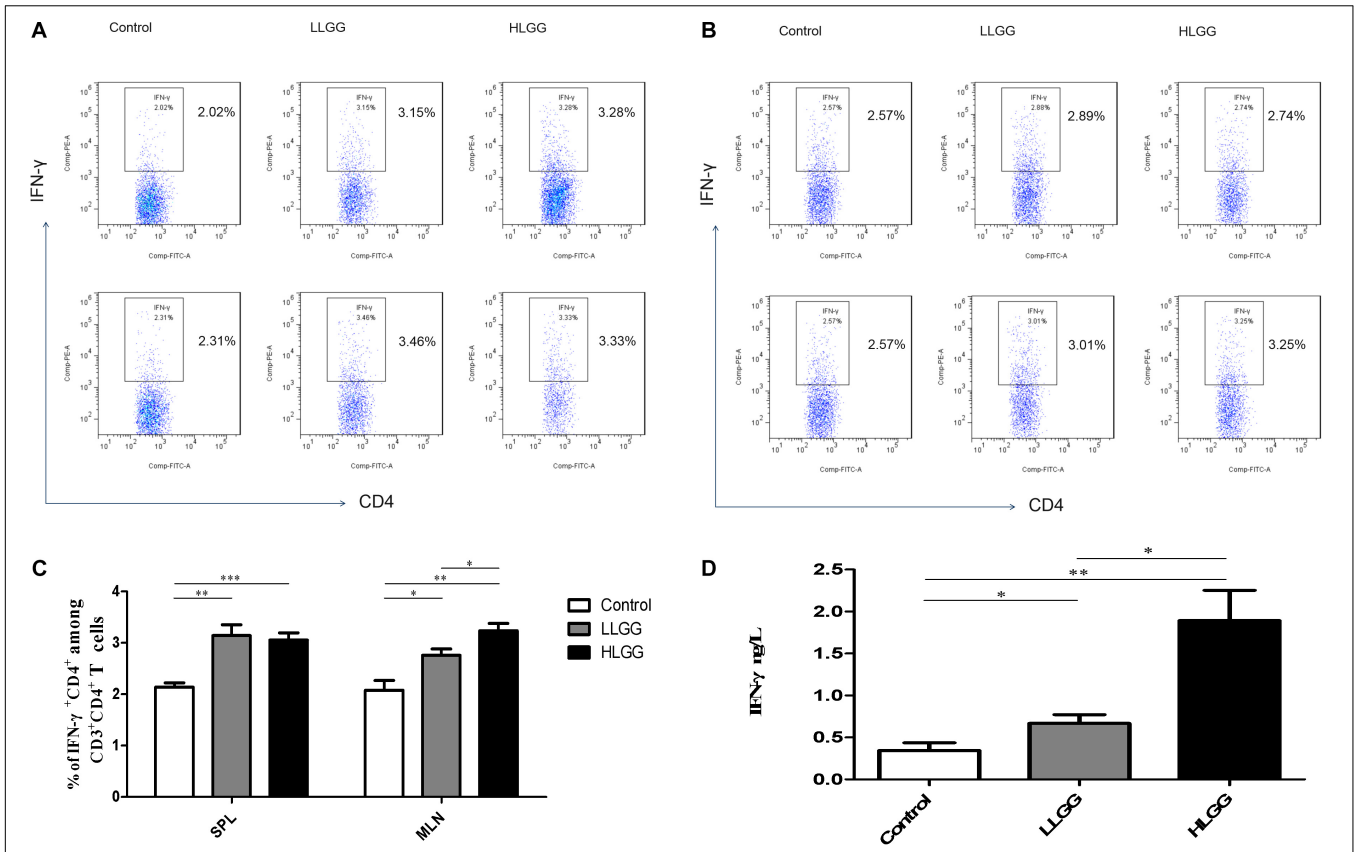

FIGURE 6 | LGG treatment increased the percentage of $CD3^+CD4^+$ IFN-γ^+ Th1 cells in secondary immunological organs and the serum IFN-γ concentration. **(A)** Changes in the proportion of $CD3^+CD4^+$IFN-γ^+ Th1 cells in the SPLs shown by representative flow cytometry data. **(B)** Changes in the proportion of $CD3^+CD4^+$IFN-γ^+ Th1 cells in the MLNs shown by representative flow cytometry data. **(C)** Statistical data showing the percentage of $CD3^+CD4^+$ IFN-γ^+ Th1 cells in the SPLs and MLNs from **(A,B)**. **(D)** Serum IFN-γ levels in mice treated with LGG as detected by ELISA. *$0.01 < P \leq 0.05$, **$0.001 < P \leq 0.01$, ***$P \leq 0.001$.

and 2.900 ± 0.223%, respectively. Compared to the percentage of $CD3^+CD4^+$IL-4^+ Th2 cells in the MLNs from the control group, a higher percentage was observed in the LLGG group ($p < 0.05$, $p = 0.0212$) and HLGG group ($p < 0.05$, $p = 0.0076$) (**Figures 7B,C**). Compared to the control group, the percentage of Th2 cells was decreased in both the LLGG and HLGG groups.

After 7 LGG interventions, the serum IL-4 concentration was detected by ELISA. Compared to the concentration of serum IL-4 observed in the control group, a higher concentration was observed in the LLGG group ($p < 0.05$, $p = 0.0472$) and HLGG group ($p < 0.05$, $p = 0.0032$). The concentration of serum IL-4 in the HLGG group was also higher than that observed in the LLGG group ($p < 0.05$, $p = 0.0117$) (**Figure 7D**).

IFN-γ can promote the differentiation of Th0 cells into Th1 cells and affect the ratio of Th1/Th2, while IL-4 can promote the differentiation of Th0 cells into Th2 cells. Th1 cells primarily mediate immune responses related to cellular and local inflammation and participate in cellular immunity. The primary function of Th2 cells is to stimulate B cell proliferation and produce antibodies, which are involved in humoral immunity. After LGG intervention, the ratio of IFN-γ/IL-4 expressed by $CD4^+$ T cells in the SPLs and MLNs was greater than 1 (**Supplementary Figures 4A,B**). The ratio of IFN-γ/IL-4 in serum detected by ELISA was also greater than 1 (**Supplementary Figure 4C**). These results suggest that LGG tends to mediate the Th2 immune response.

Effects of LGG on Th17 Cell Differentiation in Mice

Th17 cells represent a small proportion of $CD4^+$ T cells that express IL-17. After 7 LGG interventions, the percentages of $CD3^+CD4^+$ IL-17^+ Th17 cells in the SPLs from the control, LLGG and HLGG groups were 3.104 ± 0.038%, 5.144 ± 0.44%, and 4.656 ± 0.16%, respectively. Compared to the percentage of $CD3^+CD4^+$IL-17^+ Th17 cells in the SPLs from the control group, a higher percentage was observed in the LLGG group ($p < 0.05$, $p = 0.0018$) and HLGG group ($p < 0.05$, $p = 0.0001$) (**Figures 8A,C**). The percentages of $CD3^+CD4^+$IL-17^+ Th17 cells in the MLNs from the control, LLGG and HLGG groups were 4.003 ± 0.17%, 5.390 ± 0.42%, and 6.528 ± 0.40%, respectively. Compared to the percentage of $CD3^+CD4^+$IL-17^+ Th17 cells from the MLNs in the control group, a higher percentage was observed in the LLGG group ($p < 0.05$, $p = 0.0242$) and HLGG group ($p < 0.05$, $p = 0.0012$) (**Figures 8B,C**). No significant difference was observed in

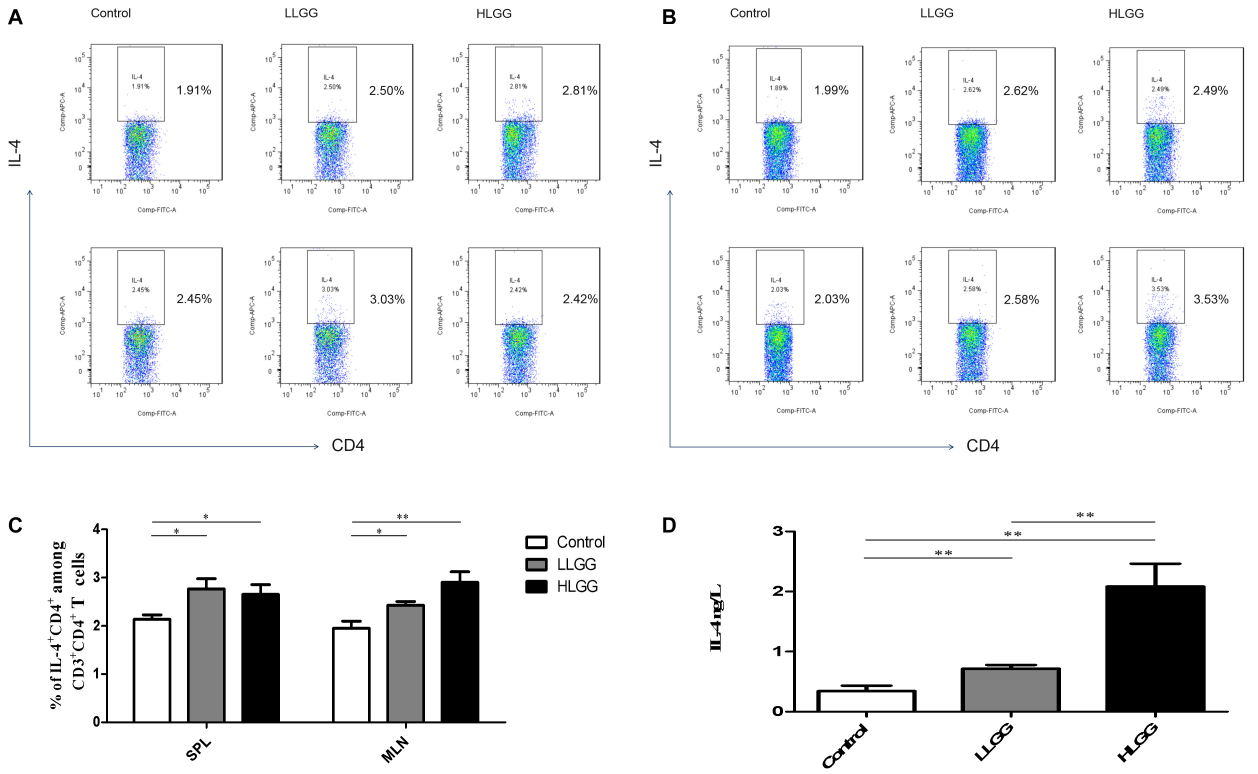

FIGURE 7 | LGG treatment increased the percentages of $CD3^+CD4^+IL\text{-}4^+$ Th2 cells in secondary immunological organs and the serum IL-4 concentration. **(A)** Changes in the proportion of $CD3^+CD4^+IL\text{-}4^+$ Th2 cells in the SPLs shown by representative flow cytometry data. **(B)** Changes in the proportion of $CD3^+CD4^+IL\text{-}4^+$ Th2 cells in the MLNs shown by representative flow cytometry data. **(C)** Statistical data showing the percentage of $CD3^+CD4^+$ $IL\text{-}4^+$ Th2 cells in the SPLs and MLNs from **(A,B)**. **(D)** Serum IL-4 levels in mice treated with LGG as detected by ELISA. *$0.01 < P \leq 0.05$, **$0.001 < P \leq 0.01$.

the percentage of $CD3^+CD4^+IL\text{-}17^+$ Th17 cells between the LLGG and HLGG groups. Compared to the control group, both the LLGG and HLGG groups showed an increase in the percentage of Th17 cells.

Effect of LGG on CD4+CD25+Foxp3+ Treg Subsets in Mice

Treg cells express CD4 and CD25, as well as the transcription factor Foxp3, which controls their development. After 7 LGG interventions, the percentages of $CD4^+CD25^+Foxp3^+$ Treg cells in the SPLs from the control, LLGG and HLGG groups were 11.23 ± 0.05%, 11.62 ± 0.13%, and 11.16 ± 0.09%, respectively. The mice in the LLGG group had a higher percentage of $CD4^+CD25^+Foxp3^+$ Treg cells in the SPL than those in the control group ($p < 0.05$, $p = 0.0311$) and HLGG group ($p < 0.05$, $p = 0.0251$) (**Figures 9A,D**). The percentages of $CD4^+CD25^+Foxp3^+$ Treg cells in the MLNs from the control and HLGG groups were 9.896 ± 0.1913% and 9.314 ± 0.16%, respectively. Compared to the percentage of $CD4^+CD25^+Foxp3^+$ Treg cells observed in the MLNs from the control group, a lower percentage was observed in the MLNs from the HLGG group ($p < 0.05$, $p = 0.0492$) (**Figures 9B,D**). The percentages of $CD4^+CD25^+Foxp3^+$ Treg cells in PPs from the control, LLGG and HLGG groups were 8.898 ± 0.17%, 8.194 ± 0.09%, and 10.05 ± 0.20%, respectively. Compared to the percentage of $CD4^+CD25^+Foxp3^+$ Treg cells in PPs from the control group, a lower percentage was observed in the LLGG group ($p < 0.05$, $p = 0.0069$), while a higher percentage was observed in the HLGG group ($p < 0.05$, $p = 0.0028$). The percentage of $CD4^+CD25^+Foxp3^+$ Treg cells in PPs from the HLGG group was significantly higher than that observed in the LLGG group ($p < 0.05$, $p < 0.0001$) (**Figures 9C,D**). Compared to the control group, the LLGG group exhibited an increased percentage of Treg cells in the SPLs and a decreased percentage of Treg cells in the MLNs and PPs, while the HLGG group had a decreased percentage of Treg cells in the MLNs and an increased percentage of Treg cells in the PPs.

In summary, LGG could affect the differentiation of Th1, Th2, Th17, and Treg cells in peripheral lymphoid organs (SPLs) and intestinal secondary lymphoid tissues (MLNs and PPs) of mice. LGG could promote the differentiation of Th0 cells into Th1, Th2, and Th17 cells and enhance the immunity of mice. The effect of the high-dose LGG treatment was stronger than that of the low-dose treatment. The LLGG treatment could significantly increase the proportion of Treg cells in the SPL and reduce that observed in PPs, while the HLGG treatment could significantly increase the proportion of Treg cells in PPs and reduce that observed in the MLNs, thereby regulating the balance between tolerance and immunity.

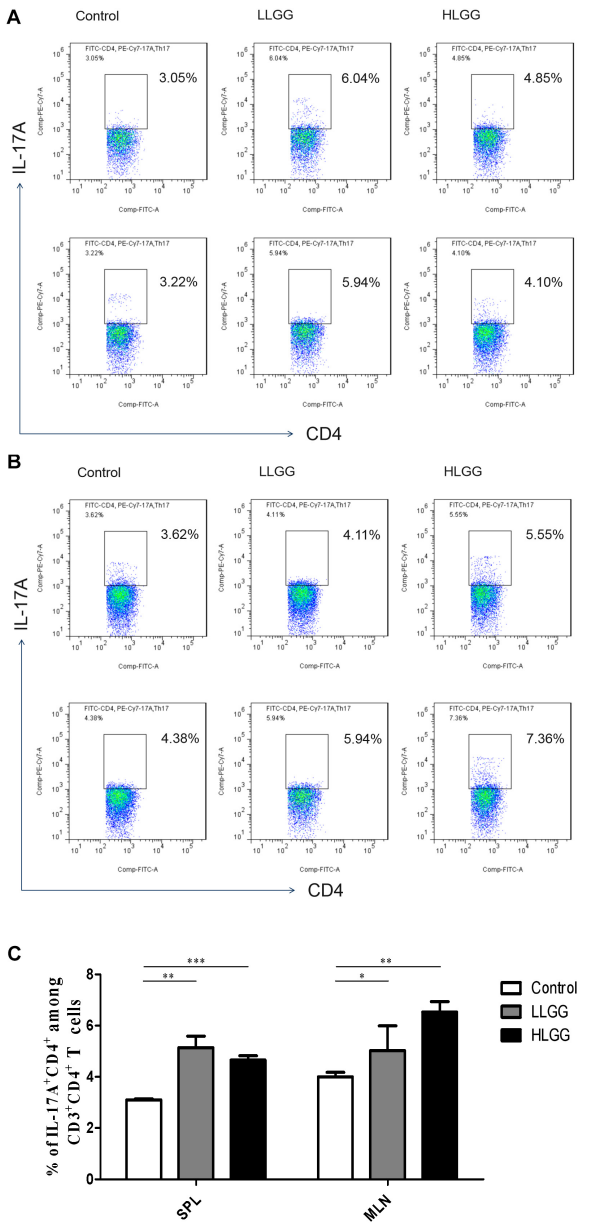

FIGURE 8 | LGG treatment increased the percentage of $CD3^+CD4^+IL\text{-}17^+$ Th17 cells in secondary immunological organs. **(A)** Changes in the proportion of $CD3^+CD4^+IL\text{-}17^+$ Th17 cells in the SPLs shown by representative flow cytometry data. **(B)** Changes in the proportion of $CD3^+CD4^+IL\text{-}17^+$ Th17 cells in the MLNs shown by representative flow cytometry data. **(C)** Statistical data showing the percentage of $CD3^+CD4^+IL\text{-}17^+$ Th17 cells in the SPL and MLN from **(A,B)**. *$0.01 < P \leq 0.05$, **$0.001 < P \leq 0.01$, ***$P \leq 0.001$.

DISCUSSION

The animal intestinal tract is inhabited by a large number of microflora, which establish a stable and mutually beneficial symbiotic relationship between themselves and the host and maintain a dynamic balance in the intestinal tract. Previous studies have shown that *Lactobacillus* can colonize the intestinal tract, regulate the intestinal flora, increase the number of the dominant intestinal flora, improve intestinal health, and effectively prevent the infection of pathogens. Based on high-throughput sequencing analysis, LGG ATCC53103 fed to gnotobiotic pigs was shown to maintain intestinal balance and increase the dominant intestinal flora, especially to maintain the basal level of enterococci and effectively resist rotavirus infection (Zhang et al., 2014). Similarly, the results of the present study further confirmed the effect of LGG ATCC53103 on the intestinal flora in mice. After the oral administration of LGG to BALB/c mice at a high-dose (HLGG group) and low-dose (LLGG group), the observed OTU, Chao1, ACE, Simpson, and Shannon indices were obtained according to the sequencing results. Both the LLGG and HLGG treatments increased the observed OTU, Chao1, ACE, and Shannon indices and decreased the Simpson index, indicating that LGG can promote the abundance and diversity of the intestinal flora. LGG increases the number of *Firmicutes*, most of which are gram-positive beneficial bacteria, while decreasing the number of *Proteobacteria*, which are mostly gram-negative intestinal pathogens, providing intestinal probiotics a dominant community position in mice in. In addition, the results showed that the abundance and diversity of the intestinal flora in the LLGG group was higher than that observed in the HLGG group. LAB in the intestinal tract primarily regulate the balance of the intestinal flora through occupation, competition, secretion and pH value regulation. Excessive LAB will also produce conditions that are not conducive to the growth of probiotics, such as too low of a pH value, making it is necessary to further study the optimal dose of LGG to better promote the abundance and diversity of the intestinal flora.

In the present study, we showed that LGG can increase the number of $CD4^+$ T lymphocytes in a dose-dependent manner, contributing to the differentiation of Th cells and enhancing cellular immune function. Th1 cells primarily secrete IFN-γ, IL-2, and TNF-α, which can enhance the cell-mediated immune response, while Th2 cells primarily secrete IL-4, IL-5, and IL-13 to enhance the humoral immune response. Th1/Th2 cells maintain a dynamic balance and can maintain normal cellular and humoral immune responses. Some studies have shown that LAB can affect the Th1/Th2 immune response (Borchers et al., 2002). When we analyzed the immune responses of mice treated with LGG, we observed that LGG could promote the expression and secretion of IFN-γ by Th1 cells and of IL-4 by Th2 cells. The ELISA results showed that LGG promoted the secretion of IFN-γ and IL-4 in serum in a dose-dependent manner, which further confirmed the flow cytometry results. The analysis of the ratio of IFN-γ/IL-4 between the LGG and control groups showed that the ratio of IFN-γ/IL-4 in the LGG group was more than 1, and there was a significant difference between the two groups ($p < 0.05$), suggesting that LGG tends to mediate the Th1 immune response. In addition, the results of the present study showed that LGG upregulated the expression of IL-17 in $CD4^+$ T cells. An increase in IL-17 promotes the ability of the host to resist infection by bacteria, mycobacteria and fungi (Curtis and Way, 2009). LGG can promote the differentiation of Th0 cells into Th17 cells, promote the expression of IL-17, regulate immunity and enhance

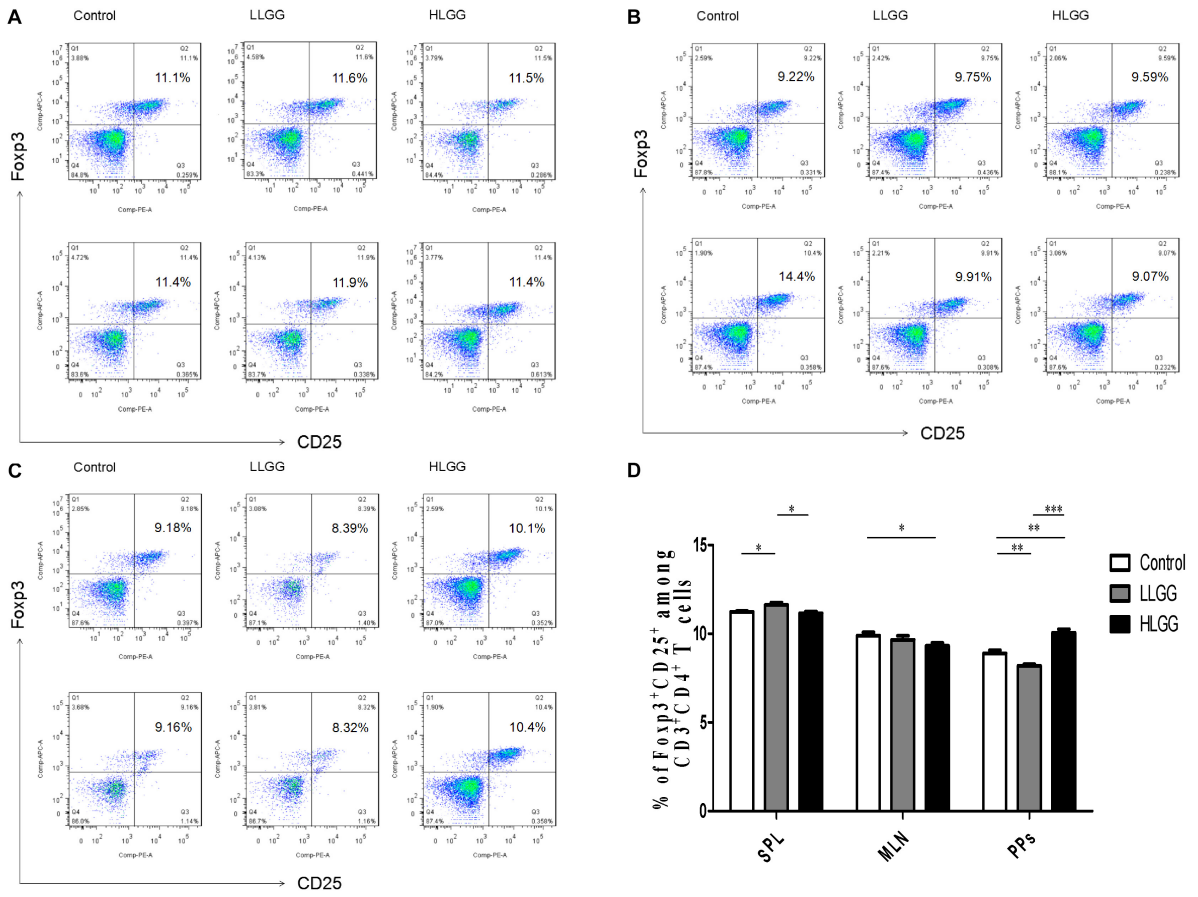

FIGURE 9 | LGG treatment affected the percentage of $CD4^+CD25^+Foxp3^+$ Treg cells in secondary immunological organs. **(A)** Changes in the proportion of $CD4^+CD25^+Foxp3^+$ Treg cells in the SPLs shown by representative flow cytometry data. **(B)** Changes in the proportion of $CD4^+CD25^+Foxp3^+$ Treg cells in the MLN shown by representative flow cytometry data. **(C)** Changes in the proportion of $CD4^+CD25^+Foxp3^+$ Treg cells in PPs shown by representative flow cytometry data. **(D)** Statistical data showing the percentage of $CD4^+CD25^+Foxp3^+$ Treg cells in the SPLs, MLNs, and PPs from **(A–C)**. *$0.01 < P \leq 0.05$, **$0.001 < P \leq 0.01$, ***$P \leq 0.001$.

the defenses of the host. We demonstrated that LGG can also regulate the differentiation of Treg cells. Chen et al. showed that LGG can significantly improve the differentiation and promote the balance of Th-17 and Treg cells as well as alleviate alcohol-induced liver injury (Chen et al., 2016). These results indicated that LGG can not only regulate the balance between Th1 and Th2 but also regulate the balance between Th-17 and Treg. By regulating the host immune status, LGG may improve the host defense against pathogenic microbial infection, which can be evaluated by an infectious model in the future.

To sum up, compared with HLGG, LLGG can better improve the abundance and diversity of intestinal flora, but HLGG group can promote the number and function of T lymphocytes more than LLGG group. Other studies have shown that a mixture of *Lactobacillus* and *Bifidobacterium* at low concentrations ($<1 \times 10^6$ CFU/mL) enhances IFN-γ production and inhibits IL-4 production in mitogen activated mouse and human spleen T cells, while high concentrations ($\geq 1 \times 10^6$ CFU/mL) inhibit mitogen-induced T cell proliferation (Li et al., 2011). In this study, this phenomenon is related to the dose of LGG. We need to further study the optimal dose of LGG to maximize the abundance and diversity of intestinal flora and make the host have the best immunity. Lactic acid bacteria are increasingly used to improve human health, relieve disease symptoms, and improve the efficacy of vaccines. Our results show that the more probiotics, not always the better. It may be harmful if the optimal dose is not used for the appropriate purpose. The importance of dose selection should be emphasized in the research of probiotics in the future. The mechanism of dose effect of probiotics needs to be further studied.

MATERIALS AND METHODS

Bacterial Strains

LGG ATCC 53103 was grown in De Man, Rogosa, and Sharpe (MRS) broth for 12 h at 37°C. After culturing overnight, the bacteria were inoculated 1:100 in fresh MRS broth and grown under anaerobic conditions until reaching the mid-log phase. Then, the colonies were counted, and the cell density was adjusted to 5×10^9 colony forming units (CFU)/ml.

Animal Experimental Procedure for LGG Intervention

Forty-five 6-week-old female BALB/c mice were randomly divided into 3 groups, with 15 mice in each group. The mice in the normal control group (Control) were intragastrically administered PBS (200 μL) every other day for 7 total treatments. The mice in the low-dose LGG group (LLGG) were intragastrically administered LGG every other day for 7 total treatments, with intervention doses of 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, and 10^9 CFU. The mice in the high-dose LGG group (HLGG) were intragastrically administered 10^9 CFU every other day for 7 total treatments. One week after the LGG interventions (on the 21st day), the 45 from the three groups were sacrificed.

DNA Extraction, PCR Amplification, 16S rRNA Sequencing

Total genome DNA from samples was extracted using CTAB method. DNA concentration and purity was monitored on 1% agarose gels. According to the concentration, DNA was diluted to 1 ng/μL using sterile water. 16S rRNA genes of distinct regions (16S V3-V4) were amplified used specific primer (341F: 5′-CCTAYGGGRBGCASCAG-3′ and 806R: 5′-GGACTACNNGGGTATCTAAT-3′) with the barcode. All PCR reactions were carried out with 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 2 μM of forward and reverse primers and about 10 ng template DNA. Thermal cycling consisted of initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, elongation at 72°C for 30 s, and finally 72°C for 5 min. Mix same volume of 1X loading buffer (contained SYB green) with PCR products and operate electrophoresis on 2% agarose gel for detection. PCR products was mixed in equidensity ratios. Then, mixture PCR products was purified with Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, United States) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Fisher Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina NovaSeq platform and 250 bp paired-end reads were generated.

Bioinformatics and Sequencing Data Analysis

After sequencing, paired-end reads was assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH (V1.2.7)[1] (Magoč and Salzberg, 2011). Quality filtering on the raw tags were performed under specific filtering conditions to obtain the high-quality clean tags (Bokulich et al., 2013) according to the QIIME (V1.9.1)[2] (Caporaso et al., 2010)quality controlled process. The tags were compared with the reference database (Silva database)[3] using UCHIME algorithm[4], (Edgar et al., 2011) to detect chimera sequences, and then the chimera sequences were removed (Haas et al., 2011). Then the Effective Tags finally obtained. Sequences analysis were performed by Uparse software (Uparse v7.0.1001)[5] (Edgar, 2013). Sequences with ≥97% similarity were assigned to the same OTUs. Representative sequence for each OTU was screened for further annotation. For each representative sequence, the Silva Database (see text footnote 3) (Quast et al., 2013) was used based on Mothur algorithm to annotate taxonomic information. Alpha diversity is applied in analyzing complexity of species diversity for a sample through 4 indices, including Chao1, Shannon, Simpson, and ACE. All this indices in our samples were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3). Heatmap and network analysis were performed to visualize the taxon abundance at the genus level using graphviz software (version 2.38.0) and R software (Version 2.15.3), respectively. Linear discriminant analysis (LDA) effect size (LEfSe) was used to detect significant changes in relative abundance of microbial taxa among different groups. A significance value of less than 0.05 and an LDA effect size of greater than 3 were used as thresholds for the LEfSe analysis. Multivariate data analysis, including Venn analysis of shared and unique OTUs and partial least squares-discriminant analysis (PLS-DA), was performed using R and Simca-P 12.0 (Umetrics, Umeå, Sweden), respectively. The raw reads were deposited into the NCBI Sequence Read Archive database (accession: PRJNA 675996).

Collection of Tissue Samples and Preparation of Single Cell Suspensions for Flow Cytometry

The spleen, mesenteric lymph node and Peyer's patches of mice were dissected out using ophthalmic scissors and forceps and placed in a 200-mesh aseptic filter, which was placed in a sterile plate with 1 mL of RPMI-1640 culture medium. The tissue was gently and fully ground with the end of a sterile 1-mL syringe, transferred to an aseptic 1.5-mL EP tube with a pipette, and centrifuged at 4°C and 2,000 rpm for 5 min, after which the supernatant was discarded, and lymphocytes were collected. The lymphocytes were wash once with FACS buffer, resuspended in 500 μL of red blood cell lysate, and lysed at room temperature for 2 min. Then, 500 μL of RPMI-1640 culture medium was added to stop lysis, after which the samples were centrifuged at 4°C and 2,000 rpm for 5 min, and the supernatant was discarded. The lymphocytes were then collected, washed with FACS buffer twice, resuspended in 1 mL PBS and counted with a cell counting chamber.

A 10-cm section of small intestine harboring Peyer's patches was placed in cold PBS (without Ca^{2+} and Mg^{2+}). Then, the fat was removed, and the small intestine was cut longitudinally. Subsequently, the intestinal tissue was rinsed with precooled PBS (without Ca^{2+} and Mg^{2+}) at 4°C until clean, after which it was transversely cut into intestinal segments 0.5∼1 cm in

[1]http://ccb.jhu.edu/software/FLASH/

[2]http://qiime.org/scripts/split_libraries_fastq.html

[3]https://www.arb-silva.de/

[4]http://www.drive5.com/usearch/manual/uchime_algo.html

[5]http://drive5.com/uparse/

length. The intestinal segments were then placed in 5 mL of intraepithelial lymphocyte (IEL) separation solution (10 mM DTT, 2 mM EDTA, and 3% FBS in RPMI 1640 medium) and incubated at a constant temperature (37°C) with shaking (200 r/min) for 15 min. Subsequently, the intestinal segments were passed through a 200-mesh nylon filter and then added to 5 mL of IEL separation solution, after which the above filtration procedure was repeated after shaking at 37°C for 15 min. The remaining intestinal segments were placed in 5 mL of lamina propria (LP) digestion solution (1.5 g/L collagenase IV, 3 mg/L neutral protease (Dispase), 100 kU/L DNase I, and 5% FBS in RPMI 1640 medium). After shaking (200 r/min) at a constant temperature (37°C) for 45 min, the remaining intestinal segments were filtered with a 300-mesh aseptic filter, and the solid residue was discarded, with the filtrate collected in a 15-mL aseptic tube. After centrifugation at 4°C and 400 × *g* for 10 min, the supernatant was discarded to collect the LP cells. Then, 4 mL of an 80% isotonic Percoll solution was placed at the bottom of a 15 mL centrifuge tube, and the LP cell pellets were resuspended in 7 mL of a 40% isotonic Percoll solution. The resuspended LP cell pellets were fully mixed, pipetted onto the 80% isotonic Percoll solution, and then centrifuged at 20°C and 2,300 rpm for 20 min. Subsequently, the upper liquid layer was discarded, and the cells between two liquid levels were transferred to a new 15-mL centrifuge tube, after which PBS (without Ca^{2+} and Mg^{2+}) was added to a volume of 15 mL. After centrifugation at 4°C and 2,000 rpm for 8 min, the supernatant was discarded. Then, the remaining cell pellets were washed twice with PBS (without Ca^{2+} and Mg^{2+}), resuspended and counted.

Flow Cytometry

For flow cytometry analysis, the following antibodies were used: PerCP-Cy5.5-B220 (clone: RA3-6B2), PerCP-CD3 (clone: 17A2), phycoerythrin (PE)-CD3 (clone: 17A2), fluorescein isothiocyanate (FITC)-CD4 (clone: GK1.5), allophycocyanin (APC)-CD8 (clone: 53-6.7), PE-CD25 (clone: PC61), PE-IFN-γ (clone: XMG1.2), APC-IL-4 (clone: 11B11), PE-Cy7-IL-17 (clone: TC11-18H10), and APC-Foxp3 (clone: MF23). All antibodies were purchased from BD Biosciences (San Jose, CA, United States).

For intracellular cytokine staining, 1∼2 × 10^6 single cell suspensions were seeded into the 48-well cell culture plate, and 0.5 mL of complete medium was added to each well. Each well was incubated with 20 ng/mL of phorbol 12-myristate 13-acetate (PMA; Sigma) and 1 μg/mL ionomycin (Sigma) at 37°C for 4 h, after which GolgiStop (BD Biosciences) was added and the samples were incubated for 2 h. Then, the cells were resuspended in 100 μL PBS (without Ca^{2+} and Mg^{2+}) and incubated with 10 μL of specific CD3 and CD4 monoclonal fluorescent antibodies at room temperature for 30 min away from light. Subsequently, the cells were washed twice with the proper amount of PBS (without Ca^{2+} and Mg^{2+}) and centrifuged at 4°C and 2,000 rpm for 5 min, after which the supernatant was discarded. Then, the cell pellets were mixed well with 250 μL of fixation/permeabilization solution and incubated at 4°C for 20 min. One milliliter of 1 × BD Perm/Wash™ buffer was directly added to each tube, and after centrifugation at 4°C and 2,000 rpm for 5 min, the cells were washed twice, and resuspended in a volume of 100 μL. Subsequently, 10 μL monoclonal fluorescent antibodies against IFN-γ, IL-4, and IL-17 was added to each sample tube and incubated at 4°C for 30 min after mixing. Each tube was then washed twice with the proper amount of PBS (without Ca^{2+} and Mg^{2+}), leaving 300 μL of buffer to resuspend the cells. Subsequently, the cell suspension was passed through a 300-mesh nylon filter membrane into the flow and detected on an LSR-FORTESA flow cytometer (BD Biosciences).

For $CD4^+CD25^+Foxp3^+$ Treg staining, 1 × 10^6 single cell suspensions were incubated with 10 μL of specific monoclonal fluorescent antibodies against CD4 and CD25 and at room temperature without light for 30 min. Then, 1 mL of freshly prepared 1 × Fix/Perm Buffer working solution was directly added to each tube. The tubes were mixed by shaking for 3 s and then incubated at 4°C without light for 40 min. The fixed and permeabilized cells were resuspended in 1 mL of 1 × Perm/Wash Buffer, centrifuged at 4°C and 350 × *g* for 6 min, and then the supernatant was discarded. The cell pellets were resuspended in 2 mL 1 × Perm/Wash Buffer and centrifuged at 4°C and 350 × *g* for 6 min, and the supernatant was discarded. The cells were then resuspended in 100 μL of 1 × Perm/Wash Buffer, mixed with an appropriate amount of specific anti-Foxp3 antibody, vortexed for 10 s to mix well and then incubated at 4°C without light for 40 min. Each tube was then washed twice with 2 mL of 1 × Perm/Wash Buffer and centrifuged at 4°C and 350 × *g* for 6 min, and the supernatant was discarded. The cell pellets were then resuspended in 300 μL of PBS (without Ca^{2+} and Mg^{2+}), passed through a 300-mesh nylon filter membrane into a tube and detected on an LSR-FORTESA flow cytometer (BD Biosciences). The data were analyzed using FlowJo 9.3 (Tree Star, Ashland, OR, United States).

Detection of IFN-γ and IL-4 in Serum by ELISA

The IFN-γ and IL-4 levels in serum were detected using a commercial ELISA kit (R&D SYSTEMS) following the manufacturer's instructions. Briefly, the standard was diluted and loaded according to the manufacturer's instructions, and the blank and sample wells were appropriately prepared. On the precoated plate, 50 μL of the diluted sample solution was added to the well of the experimental sample to be tested. The sample was added to the bottom of the plate well without touching the well wall, after which the samples were gently shaken to mix well. The sealed plate was ten incubated at 37°C for 30 min. The sealing film was then carefully removed, the was liquid discarded, and each well was filled with wash buffer and incubated for 30 s and before discarding the liquid, with the process repeated 5 times. Then, 50 μL of enzyme-labeled reagent was added to each well, except for the blank well, incubated at 37°C for 30 min and then the wells were washed five times. Subsequently, 50 μL of chromogenic agents A and B were added to each well, mixed by gently by shaking and then incubated at 37°C for 15 min without light. Then, 50 μL of terminator solution was added to each well to terminate the reaction. The plate was placed into a microplate reader, the blank well was adjusted to zero, and the absorbance of each well was measured at 450 nm.

Statistical Analysis

The data were analyzed using GraphPad Prism 5.0. Student's t-test was used to compare two groups, and one-way ANOVA was used to analyze more than two groups. Differences were considered significant when $p < 0.05$.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s. The raw reads for 16S rRNA Sequencing were deposited into the NCBI Sequence Read Archive database (accession: PRJNA 675996).

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethics Committee of Jilin Agricultural University.

AUTHOR CONTRIBUTIONS

CS, XC, GY, and CW conceived and designed the experiments and wrote the manuscript. CS, XY, YL, MC, and RW performed the experiments. MC, YJ, DZ, JW, and HY performed high through sequencing analysis. CS, YZ, WY, HH, and LY performed the flow cytometry analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.607735/full#supplementary-material

Supplementary Figure 1 | The curves of the OTUs obtained from 15 samples. **(A)** Rarefaction curves. **(B)** Shannon curves. **(C)** Coverage curves. **(D)** OTU rank-abundance curves. Control, samples from the control group; LLGG, low-dose LGG group; HLGG, high-dose LGG group.

Supplementary Figure 2 | Two-dimensional PLS-DA score plots distinguishing the gut microbiota of BALB/c mice in response to LGG pretreatment based on the relative abundance of OTUs.

Supplementary Figure 3 | Heatmap showing the differentially abundant predicted KEGG pathways.

Supplementary Figure 4 | Effect of LGG on the IFN-γ/IL-4 ratio in the serum of mice. **(A)** The ratio of IFN-γ/IL-4 expressed by $CD4^+$ T cells in the SPL after LGG intervention. **(B)** The ratio of IFN-γ/IL-4 expressed by $CD4^+$ T cells in the MLN after LGG intervention. **(C)** The ratio of IFN-γ/IL-4 in serum.

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ORIGINAL RESEARCH
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Bactericidal Permeability Increasing Protein Deficiency Aggravates Acute Colitis in Mice by Increasing the Serum Levels of Lipopolysaccharide

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Objective: The objective of this study was to understand the role of bactericidal permeability increasing protein (BPI) in the pathogenesis of experimental murine colitis.

Methods: We used the Cre-LoxP system to generate BPI knockout (BPI KO) mice. Acute colitis was induced in BPI KO mice and wild-type (WT) mice by subjecting the mice to 5% dextran sulfate sodium (DSS). Mice were observed for symptoms of experimental colitis. The survival of BPI KO mice to infection with *Acinetobacter baumannii*, a gram-negative bacterium, was also assessed.

Results: Southern blot, RT-PCR, and western blot results showed that the 2nd and 3rd exons of the murine *Bpi* gene were knocked out systemically, confirming successful construction of the BPI KO mouse. BPI KO mice subjected to DSS showed increased symptoms of experimental colitis, increased colonic mucosal damage, increased epithelial permeability, elevated levels of serum LPS, and a disrupted fecal microbiome as compared with WT mice. Furthermore, BPI KO mice challenged intraperitoneally with *A. baumannii* died sooner than WT mice, and the total number of bacteria in the abdominal cavity, spleen, and liver was increased in BPI KO mice as compared to WT mice.

Conclusions: We successfully generated BPI KO mice. The BPI KO mice developed worse colitis than WT mice by increased colitis symptoms and colonic mucosal damage, elevated levels of serum LPS, and a disrupted microbiome. BPI could be a potential target for treatment of ulcerative colitis in humans.

Keywords: bactericidal permeability-increasing protein, antibacterial peptide, ulcerative colitis, gram negative bacteria, gene knockout

INTRODUCTION

Bactericidal permeability-increasing protein (BPI), a cationic protein with a molecular weight of 55 kDa (1, 2), is found in the neutrophils of humans, cattle, pigs, mice, and other mammals that have bactericidal and neutralizing-endotoxins [e.g., bacterial lipopolysaccharide (LPS)] function (3, 4). In humans, BPI exists not only in neutrophils, eosinophils, and fibroblasts, but also in human mucosal epithelial cells, which are involved in the composition of the intestinal mucosal barrier (5, 6). The sequence of the murine *Bpi* gene was first reported in 2005. The mRNA expression of *Bpi* in nine tissues, including the heart, liver, spleen, lung, kidney, testis, and ovary, was measured, but positive expression was found only in the testis (7). Furthermore, the gene expression of BPI increased approximately 80–100 times in mouse neutrophils 24 h after the mice were injected with LPS (8). Our lab has prepared and identified murine *Bpi* gene systemic knockout plasmids (9) which can be used in this area of research.

Ulcerative colitis (UC) is one of two diseases that comprise IBD. UC is a chronic, non-specific colitis response, and its etiology is currently unknown (10). The manifestations of UC is diarrhea, abdominal pain, weight loss, and rectal bleeding. Histological, UC is limited to the mucosa by diffuse inflammatory cell infiltration of the mucosa with basal plasmacytosis, crypt architectural and a reduction of mucus-secreting goblet cells (11).

The stool composition and mucosal antibacterial response profile, especially BPI, have been shown to be related to the course of disease in patients with newly diagnosed UC. Additionally, the mucosal gene expression of BPI was found to be a good predictor of the disease course (12). The serum of patients with UC contains a variety of autoantibodies, such as antibodies against colonic epithelial cells, colon tissue, neutrophils, endothelial cells, heat shock proteins, and pancreatic proteins. Approximately 50–70% of patients with UC have been shown to have anti-neutrophil cytoplasmic autoantibodies (ANCAs) (11). The positive rate of BPI-ANCAs in UC increase with the severity of the patient's condition (13, 14). In addition, there is genetic diversity at the 645th base of the *Bpi*. Changing this base from A to G causes the 216th amino acid of the BPI protein to change from a lysine (Lys) to glutamic acid (Glu). This mutation is associated with inflammatory bowel disease (15).

The purpose of this study was to investigate of the role of BPI in experimental colitis. Here, we constructed BPI-floxed and BPI knockout (BPI KO) mice. We show that BPI KO mice had reduced antibacterial function. Furthermore, we subjected wild-type (WT) mice and BPI KO mice to 5% dextran sulfate sodium and assessed the development of experimental colitis in these mice.

MATERIALS AND METHODS

Mice

B6Ei.129S4-Tg (Prm-cre)58Og/EiJ.129 mice (stock #007252) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) (16). The BPI floxed 129 mouse strain and the BPI knockout 129 strain mouse strain were bred at the Experimental Animal Center of Capital Medical University (Beijing, China). All animal experiments were approved by the Animal Experiment and Experimental Animal Welfare Committee of Capital Medical University (approval numbers: AEEI-2016-010 and AEEI-2019-012, respectively). The above mice and WT (129 strain) mice were housed in micro-isolated cages with filtered air under sterile conditions, free access to sterile water and autoclaved food. The mice were 8–10 weeks old and weighed 20–30 g for all experiments. During the experiment, 0.2ml pentobarbital (0.8%) was injected intraperitoneally near the time of death to alleviate the pain of the mice.

Generation of Bactericidal Permeability Increasing Protein Knockout Mice

We used the Cre/LoxP system to insert LoxP sites on both sides of the 2nd and 3rd exons of the mouse (C57BL/6J) *Bpi* gene in order to obtain the BPI conditional gene-targeting (BpiKO) vector (17). The BpiKO vector was transfected into mouse (129 strain) embryonic stem (ES) cells. After introducing the target ES cells into the blastocyst cavity of the recipient C57BL/6J mice by microinjection, the blastocysts were transplanted into pseudopregnant female mice to obtain BPI floxed heterozygous mice ($BPI^{fl/-}$). The $BPI^{fl/-}$ mice (on a 129 background) were mated with Prm-cre mice, which yielded the F1 generation of BPI KO heterozygous ($BPI^{+/-}$) mice. $BPI^{+/-}$ mice were then mated to obtain BPI KO homozygous ($BPI^{-/-}$, or BPI KO) mice. BPI floxed mice and BPI KO mice were bred for more than 10 generations. The BPI KO did not affect the reproduction of offspring, and BPI KO mice did not present with any disease.

Identification of Bactericidal Permeability Increasing Protein Knockout Mice

Southern Blot and PCR

DNA was extracted from mouse tails and digested with the restriction enzyme NcoI. The product was separated by electrophoresis and transferred to a nylon membrane. Digoxin-labelled probes prox-3f: ATCTCCACCGACCTGATTCT and prox-3r: GGCAGATGGCGTAAGAGCAT were used to hybridize to the DNA.

PCR Identification

DNA from tail or ear of mice was extracted, and primers were designed outside of the LoxP1/2 loci at both ends of the 2nd and 3rd *Bpi* gene exons. The target gene was amplified by PCR, and 1.5% gel electrophoresis and sequencing were performed.

RT-PCR Identification

Mice were injected with 200 ng LPS intraperitoneal injection (i.p.). Twenty-four hours post-injection, RNA was extracted from both the testis tissue and neutrophils isolated from the bone marrow and spleen of mice by density gradient centrifugation and lysis of erythrocytes (Solabio, Beijing, China) as reported previously (18). RNA was extracted from the colonic mucosa after the mice were subjected to 5% DSS for 5 days. One microgram of tissue-derived RNA was reverse transcribed into 20 μL of cDNA, and 1 μL of the cDNA was used for amplifying the BPI fragment with primer P1 (5'-AACGTGCGGAAATGGTCAG-3') and primer P2 (5'-CAGTT GGAGCAGATGGTGGT-3'). The products were confirmed by 1.5% gel electrophoresis and sequenced by Sangon Biotech Co., Ltd (Shanghai, China). The murine β-actin gene was amplified

using the following primers: 5'-TGGAATCCTGTGGCATCC A-3' and 5'-TAACAGTCCGCCTAGAAGCAG-3'.

Western Blot

Splenic neutrophils were isolated 24 h post-injection of 200 ng LPS by density gradient centrifugation (Solabio, Beijing, China) as reported previously (18). Mouse splenic neutrophils and testis tissue were lysed in RIPA buffer (Applygen Technology Inc., Beijing, China) with protease inhibitors (Applygen Technology Inc., Beijing, China). The protein lysate was analyzed *via* western blot to confirm that BPI was knocked out. The plasmid pET28a-muBPI36-259 was previously prepared in 2008 (17). The plasmid was delivered to Gegen Biotechnology Co., Ltd. (Beijing, China), and the mouse monoclonal antibody (IgG) against mouse BPI amino acids 36–259 was prepared by this company, which used as the primary antibody(1:1000 dilution) in western blot. The secondary antibody was a fluorescent antibody against mouse IgG (IRDye 800CW goat anti-mouse, cat #: 926-32210; LI-COR, Lincoln, Nebraska USA) (1:10,000 dilution). The Odyssey Sa Infrared Imaging System (LI-COR, Lincoln, Nebraska USA) was used to visualize the western blots.

Routine Blood Test

Retro-orbital blood (200 μl) was obtained from mice, placed in a tube with EDTAK2(SANLI China, Liuyang, Hunan, China) to prevent coagulation, and transported at 4°C to the Institute of Experimental Animals, Chinese Academy of Medical Sciences (Beijing, China) where the blood was analyzed. The instrument used for the blood cell differential counts was ABX-DX-120 (Horiba Medical, Montpellier, France).

Resistance of Mice to Lethal Doses of Bacterial Infection

Mouse survival was observed after a lethal dose of *Acinetobacter baumannii* (ATCC: BAA-2469). *A. baumannii* was cultured in a Nutrient Broth (Cat 234000; BD, Shanghai, China) in a 37°C incubator for 12 to 16 h. The bacteria were diluted with physiological saline with 5% inactivated yeast(ANGEL YEAST CO.,LTD, Yichang, Hubei, China) to a concentration of 1×10 (4) Colony-Forming Units(CFU)/ml. Each mouse was i.p. injected with 0.5 ml of the bacterial suspension. Survival of the mice was observed for 72 h post-infection, and all deaths were recorded (19).

Furthermore, to check the multiplication of bacteria for the cause of the death, *A. baumannii* challenge was diluted to 1×10^4 CFU/ml with 5% inactivated yeast. Each mouse was injected i.p. with 0.5 ml of the solution. At 16 h post-injection, the abdominal cavity was washed with 1.5 ml of sterile physiological saline and diluted from 1×10^{-3} to 1×10^{-5}. The liver and spleen were also collected to count the bacteria. After rinsing with physiological saline, the organs were put in a tube with 10 ml of physiological saline, homogenized with a super-speed tissue homogenizer (IKA, T18; IKA®-Werke GmbH & Co., Staufen, Germany) at 13,000 × g, and then the homogenate was diluted to 1×10^{-4} with physiological saline. Bacterial counts were obtained using Pour Plate Method (from https://www.bd.com). After centrifuging the remaining peritoneal fluid at 250 × g, the solution was resuspended with normal saline to count the cells in the peritoneal cavity. Twenty microliters of peritoneal fluid were collected on a glass slide. After Wright staining, phagocytes were counted using a microscope (NIKON, Ci-s; Yokohama, Kanagama, Japan) at 400 times magnification.

Dextran Sulfate Sodium-Induced Colitis Model in Mice

The mice were given 5% DSS (DSS MW: 36,000–50,000; MP Biomedicals United States, Solon, OH USA) in their drinking water for 5 days to induce a model of acute experimental colitis as reported previously (20). The mice were observed daily, and the weight, stool consistency, and presence of blood in the stool were recorded. On the 5th days drinking 5% DSS, the mice were sacrificed and the proximal end of colon was collected. The paraffin sections of proximal end of colon were prepared. Hematoxylin and eosin (H&E) and Alcian blue-periodic acid sthiff (AB-PAS) staining were used to observe the pathological damage and inflammatory cell infiltration into the colon.

Based on previously reported methods (20), During the duration of the experiment, a disease activity index (DAI) score can be assessed to evaluate the clinical progression of colitis. The DAI is the combined score of weight loss compared to initial weight, stool consistency, and bleeding. Scores are defined as follows: weight loss: 0 (no loss), 1 (1–5%), 2 (5–10%), 3 (10–20%), and 4 (>20%); stool consistency: 0 (normal), 2 (loose stool), and 4 (diarrhea); and bleeding: 0 (no blood), 1 (Hemoccult positive), 2 (Hemoccult positive and visual pellet bleeding), and 4 (gross bleeding, blood around anus). DAI can be scored daily during the duration of the DSS treatment.

Colonic pathology scores for each group were obtained as reported previously (20). H&E stained colonic tissue sections are scored by a blinded observer using a previously published system for the following measures: crypt architecture (normal, 0—severe crypt distortion with loss of entire crypts, 3), degree of inflammatory cell infiltration (normal, 0—dense inflammatory infiltrate, 3), muscle thickening (base of crypt sits on the muscularis mucosae, 0—marked muscle thickening present, 3), goblet cell depletion (absent, 0—present, 1) and crypt abscess (absent, 0—present, 1).

Immunofluorescence Microscopy

After the mice were subjected to 5% DSS for 5 days, the paraffin sections of the proximal end of colon were prepared. The colon sections were deparaffinized in xylene and rehydrated through graded ethanol. Antigen retrieval was performed in citrate buffer with 750-W microwave for 20 min. Endogenous peroxidase was blocked using 3% H_2O_2 for 1h. Nonspecific sites were blocked by using 3% BSA for 1h. The sections were incubated with mouse monoclonal IgG (Gegen Biotechnology Co., Ltd. Beijing, China) against mouse BPI amino acids 36–259, which used as the primary antibody (1:200 dilution) over night at 4°C. Then the sections were incubated with Goat anti mouse IgG (FITC labeled) (Applygen Technology Inc., Beijing, China) (1:4,000 dilution) for 1h at room temperature. At last, the slides were covered with the DAPI mounting medium (Solarbio, Beijing, China), observe with fluorescence microscope (Leica DM6000B, German) and digitally captured images. Finally, the slides were mounted with the DAPI mounting medium (Solarbio, Beijing, China) to mount

slides. Slides were visualized with the fluorescence microscope (Olympus, BX61).

Measurement of Epithelial Permeability *In Vivo*

As described previously, (21) we used blood concentration of FITC–dextran (MW 3.0–5.0 kDa, Sigma, USA) to measure colonic epithelial permeability. The mice were given 5% DSS for 5 days. Before experiments, mice were fasted for 15h, but had unlimited access to 5% DSS. At 5th day, mice were administered by gavage 0.6 mg/g body weight FITC–dextran (1×PBS containing 30 mg/ml FITC–dextran). After 4h, the mice were anaesthetized, and Retro-orbital blood was sampled into heparinized tubes. Plasma concentration of fluorescein was measured using a microplate spectrofluorimeter (SYNERGY, BIO Tek Instruments, Inc, Winooski, USA) with excitation wavelength of 485 nm and an emission wavelength of 520 nm, serially diluted FITC–dextran as standard.

Analysis of Serum Proteins

After the mice were subjected to 5% DSS for 5 days, the peripheral blood of mice was isolated, and the serum was collected. The LPS level in the serum was determined by a limulus amebocyte lysate-based assay (Xiamen Bioendo Reagents Experimental Factory Co., Ltd.Xiamen, China). An LPS Binding protein (LBP) ELISA kit (Hycult, Uden, Netherland) was used to detect the LBP levels in mouse serum.

Detection of Myeloperoxidase and Cytokine Levels

After the mice were subjected to 5% DSS for 5 days, 1 cm of the mouse proximal end of colon was added to 0.5 mL of tissue lysate (RIPA lysate and protease inhibitor; Applygen Technology Inc., Beijing, China). After homogenization and centrifugation at 12,000 × g, the supernatant was collected, and the total protein concentration was measured by BCA (Applygen Technology Inc., Beijing, China). Myeloperoxidase (MPO) levels were detected using an MPO ELISA kit (RayBiotech, Guangzhou, China), and cytokine levels were detected using a protein chip (QAM-CYT-1-1, RayBiotech, Guangzhou, China).

16S rDNA Analysis in Mouse Stool Samples

Mouse feces was collected and stored at −80°C. The samples were then sent to Sangon Biotech Co., Ltd (Shanghai, China) for detection of the bacterial flora in the stool samples. E.Z.N.ATM Mag-Bind Soil DNA Kit (OMEGA, Shanghai, China) was used to extract DNA from mouse feces. Primer 341F: CCCTACAC GACGCTCTTCCGATCTG (barcode) CCTACGGGNGGCW GCAG and primer 805R: GACTGGAGTTCCTTGGCACCCG AGAATTCCAGACTACHVGGGTATCTAATCC were used to amplify V3–V4 region of DNA of the bacterial flora. Then sequencing and data analysis were finished by Sangon Biotech Co., Ltd (Shanghai, China).

Statistical Analysis

All data are expressed as the mean ± standard deviation (SD) and were analyzed using GraphPad Prism, version 6.01 (San Diego, CA, USA). Statistical comparisons were performed using an unpaired *t* test and Chi squared test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ were considered statistically significant.

RESULTS

Generation of Bactericidal Permeability Increasing Protein Knockout Mice

We inserted LoxP sites on both sides of the 2nd and 3rd exons of the mouse *Bpi* gene to obtain the BpiKO vector (**Figure 1**, top). The BpIKO vector was transfected into ES cells. The target ES cells were then transplanted into pseudopregnant female mice to obtain BPI$^{fl/-}$ mice (**Figure 1**, middle). BPI$^{fl/-}$ mice on a 129 background were mated with Prm-cre mice, and the F1 generation of BPI$^{+/-}$ mice was obtained (**Figure 1**, bottom).

Identification of Bactericidal Permeability Increasing Protein Knockout Mice

Southern blot was performed to confirm the genomic deletion in the BPI KO mice. As expected, the results showed a band in WT mice at 6.9 kb, a band in BPI$^{fl/fl}$ mice at 6.1 kb, and two bands, 6.1 kb and 4 kb, in BPI$^{+/-}$ mice (**Figure 2A**).

In 2005, it was reported that mouse testis highly expressed BPI by Northern blot (7). Our RT-PCR results confirmed that the testis of BPI$^{+/+}$ mice (including WT and BPI$^{fl/fl}$ mice) highly expressed gene fragments at approximately 400 bp. The sequencing results confirmed the expression of mRNA fragments from the 1st to 4th exons, while the BPI KO mouse expressed gene fragments at approximately 200 bp (**Figure 2B**), which was confirmed by our sequencing results to be the remaining mRNA fragment after deletion of the 2nd and 3rd exons (see online **Supplementary Figure 1**). Our Western blot results confirmed that the BPI$^{+/+}$ mouse showed strong target bands at approximately 55 kDa, and BPI$^{+/-}$ mouse testis showed weak target bands at approximately 55 kDa. The BPI$^{-/-}$ mouse had no 55 kDa band (**Figure 2C**).

In 2006, it was reported that the neutrophils of mice express BPI after LPS stimulation (13). Thus, we used LPS to stimulate BPI$^{-/-}$ and BPI$^{+/-}$ mice for 24 h. RT-PCR results showed that bone marrow-derived neutrophil precursor cells from LPS-treated BPI$^{-/-}$ showed the 200bp band, whereas BPI$^{+/-}$ mice showed the 200bp band and 400bp band (**Figure 2E**). However, BPI was not detected in the bone marrow-derived neutrophils by western blot.

Consequently, we stimulated mice with LPS for 24 h, and then isolated murine splenic neutrophils and performed western blot. The results confirmed that BPI$^{+/+}$ mice showed a band at approximately 55 kDa and BPI$^{-/-}$ mice did not show a 55 kDa band (**Figure 2D**).

In 2017, it was reported that BPI can be expressed in the intestine of mice infected with *Salmonella typhimurium* (22). As such, we extracted RNA from the colon epithelial tissue of mice undergoing experimental colitis *via* 5% DSS. The cDNA was set-up in a RT-PCR reaction with *Bpi* primers. The amplified product was resolved on a gel and resolved at the expected 200bp size band from BPI$^{-/-}$(BPI KO) mice (**Figure 2F**).

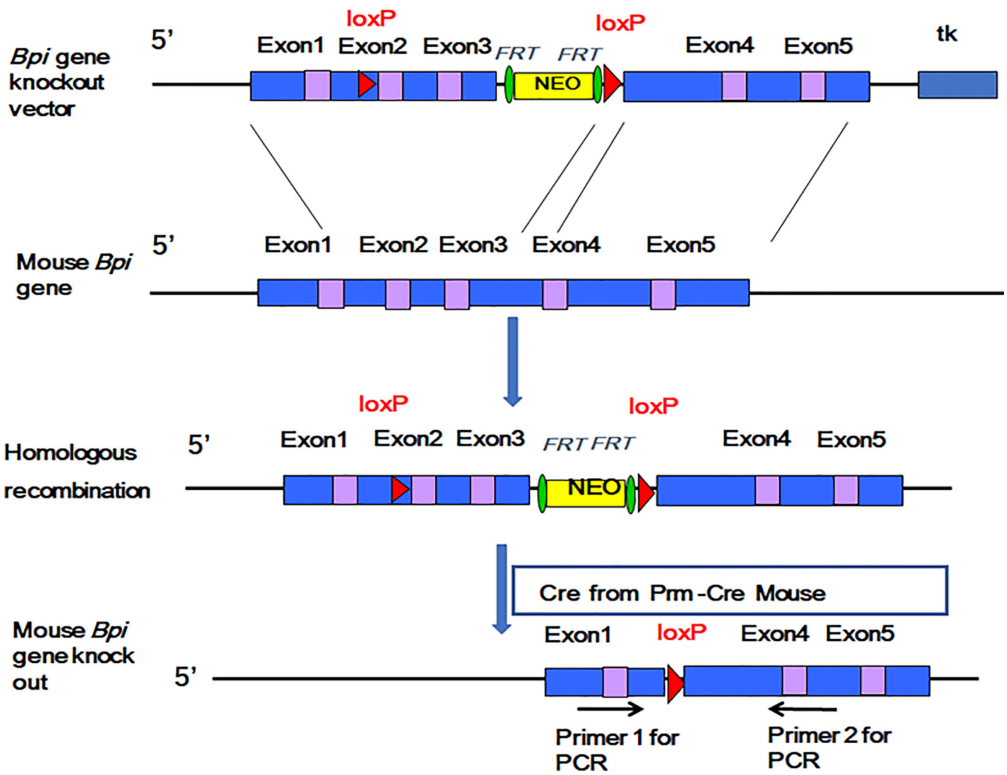

FIGURE 1 | A diagram of the construction of the BPI knockout mouse.

Neutrophil Number Was Not Affected in Bactericidal Permeability Increasing Protein KnockoutMice

WT mice and BPI KO mice underwent blood cell count. The results showed that WT mice and BPI KO mice had no statistically significant differences in the number of white blood cells, red blood cells, or neutrophils (**Figures 3A, B**).

Resistance of Mice to Lethal Doses of Bacterial Attack

In 2013, it was reported that *A. baumannii* was sensitive to BPI (23). Thus, we challenged mice with *A. baumannii* to confirm the loss of BPI function. When challenged with a lethal dose of *A. baumannii*, all BPI KOmice died (n = 10) within 72 h after injection, while 20% of the WT mice (n = 10) survived longer than 72 h (**Figure 3C**). This indicated that KO mice were more vulnerable to challenge with *A. baumannii*. After *A. baumannii* i.p. injection, the number of bacteria in the peritoneal lavage fluid of BPI KO mice was significantly higher than that of WT mice ($P < 0.001$, n = 5). The bacterial counts in the spleen and liver homogenate of BPI KO mice were significantly higher than that in WT mice ($P < 0.001$, n = 5). BPI KO mice had more peritoneal phagocytes than WT mice, but the difference was not significant (**Figure 3D**), indicating that the decreased resistance to *A. baumannii* in the BPI KO mice was not due to a reduction in phagocytes (including neutrophils).

Knockout of Bactericidal Permeability Increasing Protein Aggravated Dextran Sulfate Sodium-Induced Murine Colitis

We used 5% DSS to induce experimental colitis in mice. Both BPI KO and WT mice began to lose weight on the 3rd day after exposure to 5% DSS in their drinking water. The decrease in weight was the most obvious on the 5th day, which was significantly lower than that of control mice given regular water. When comparing the two groups of mice subjected to 5% DSS, we found that although BPI KO mice lost more weight than the WT mice, there was no significant difference between the two groups (**Figure 4A**). After three days of drinking 5% DSS, the stool of both BPI KO and WT mice was positive for rectal occult blood. The bleeding symptom was aggravated on the 4th day and aggravated on the 5th day. There was a significant difference between the two groups on the 5th day ($P = 0.039$) (**Figure 4B**). BPI KO mice and WT mice given regular drinking water were negative for rectal occult blood (data not shown). After 5 days of drinking 5% DSS, the BPI KO mice had significantly shorter colons as compared to the WT mice ($P = 0.004$) (**Figures 4C, D**). The BPI KO mice had significantly higher DAI than the WT mice (P = 0.0147) at 4th day, and (P = 0.0005) at 5th day (**Figure 4E**).

After 5 days of drinking 5% DSS, H&E and AB-PAS staining of the proximal end of colon revealed that the damage scores for the BPI KO mice were higher than those of the WT mice, with

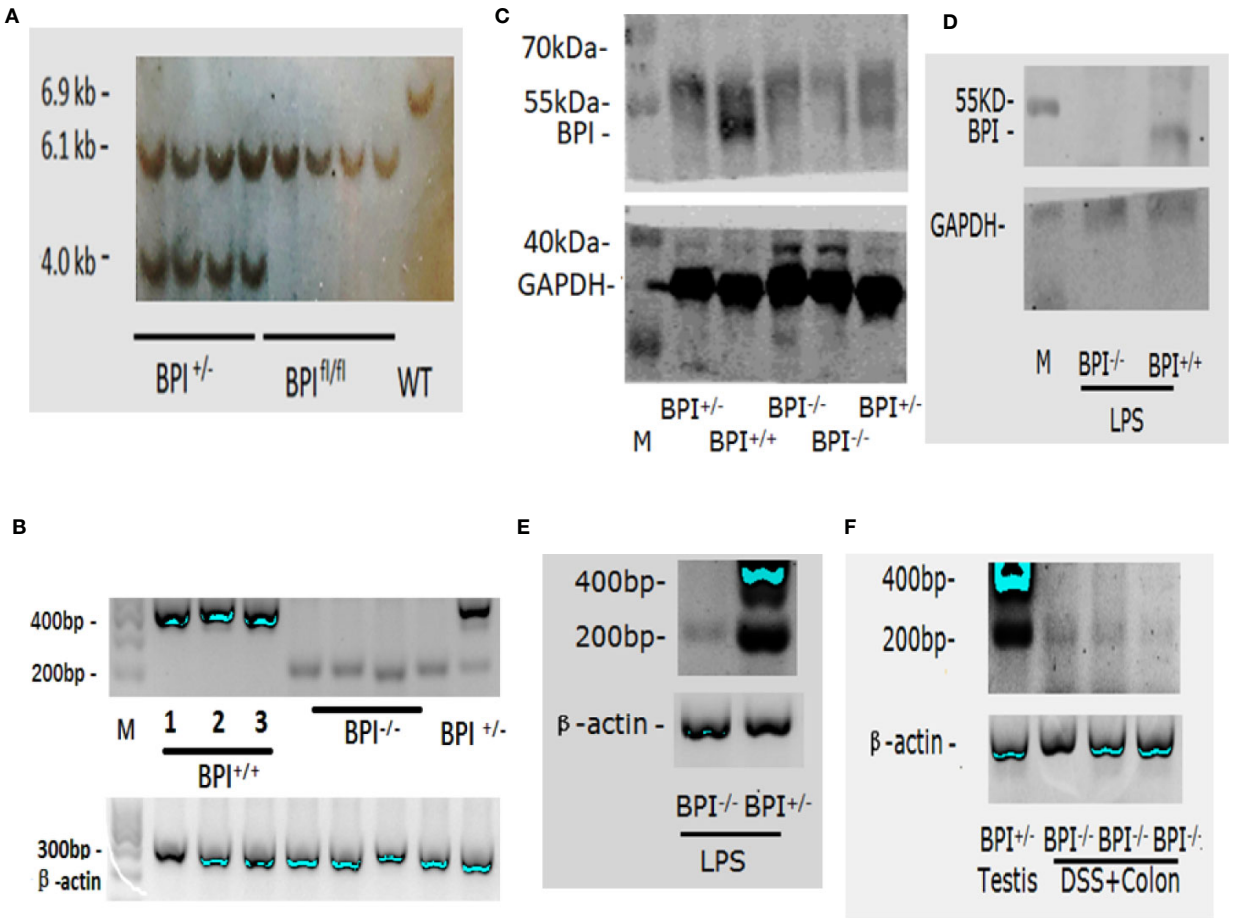

FIGURE 2 | Identification of BPI knockout mice. **(A)** Southern blot analysis of BPI KO heterozygous ($BPI^{+/-}$), BPI floxed homozygous ($BPI^{fl/fl}$), and WT mice. WT mice had a band at 6.9 kb, $BPI^{fl/fl}$ mice had a band at 6.1 kb, and $BPI^{+/-}$ mice had a band at 6.1 kb and at 4 kb. **(B)** RT-PCR identification of mouse testis tissue. $BPI^{+/+}$ mice (including WT and $BPI^{fl/fl}$) showed a band at 400 bp. BPI KO homozygous mice ($BPI^{-/-}$) mice showed a band at 200 bp. BPI KO heterozygous ($BPI^{+/-}$) mice showed both a 400 bp band and a 200 bp band. **(C)** Western blot Identification of testis tissues. $BPI^{+/+}$ mice showed a strong target band at 55 kDa. $BPI^{+/-}$ mice showed a weak target band at 55 kDa. $BPI^{-/-}$ mice did not show a band at 55 kDa. **(D)** Western blot identification of splenic neutrophils. After LPS stimulation, $BPI^{+/+}$ mice showed a target band at 55 kDa, while $BPI^{-/-}$ mice did not show a band. **(E)** After LPS stimulation, RNA from murine bone marrow-derived neutrophils was extracted and amplified by RT-PCR. $BPI^{-/-}$ mice showed a band at 200 bp, and $BPI^{+/-}$ mice showed a band at 400 bp and 200 bp. **(F)** After mice were subjected to 5% DSS for 5 days, RNA was obtained from the colonic epithelial tissue from $BPI^{-/-}$ mice and amplified by RT-PCR. A 200-bp band was amplified. RNA from $BPI^{+/-}$ mouse testis was used as a control.

significant differences in crypt curves (P = 0.0056) and inflammatory cell infiltration (P = 0.0023). The difference between the two groups was mainly within the colonic mucosa (**Figures 5A, B**). Taken together, these results suggest that a deficiency in BPI aggravates acute experimental colitis in mice.

Localization of Bactericidal Permeability Increasing Protein Protein to the Crypt of Colon

Next, we performed immunofluorescence to examine the localization of BPI. Photographs with the fluorescence microscope showed that BPI was predominantly expressed at sidewall and bottom of colon crypts in WT mice and the signal was weak. In colitis model, the level of BPI was increased at bottom of colon crypts but decreased at the sidewall of crypt which BPI deficiency was at colon of BPI KO mice (**Figure 6A**).

Bactericidal Permeability Increasing Protein Knockout Leads to an Increase in Epithelial Permeability of Colon of Dextran Sulfate Sodium-Induced Murine Colitis

Then we sought to examine whether BPI deficiency affects the intestinal epithelial permeability. On the 5th day of 5% DSS drinking, plasma concentration of fluorescein was measured at 4h after gavage FITC–dextran. The plasma FITC-dextran levels in the BPI KO mice (3.561 ± 0.4185, n = 3) were

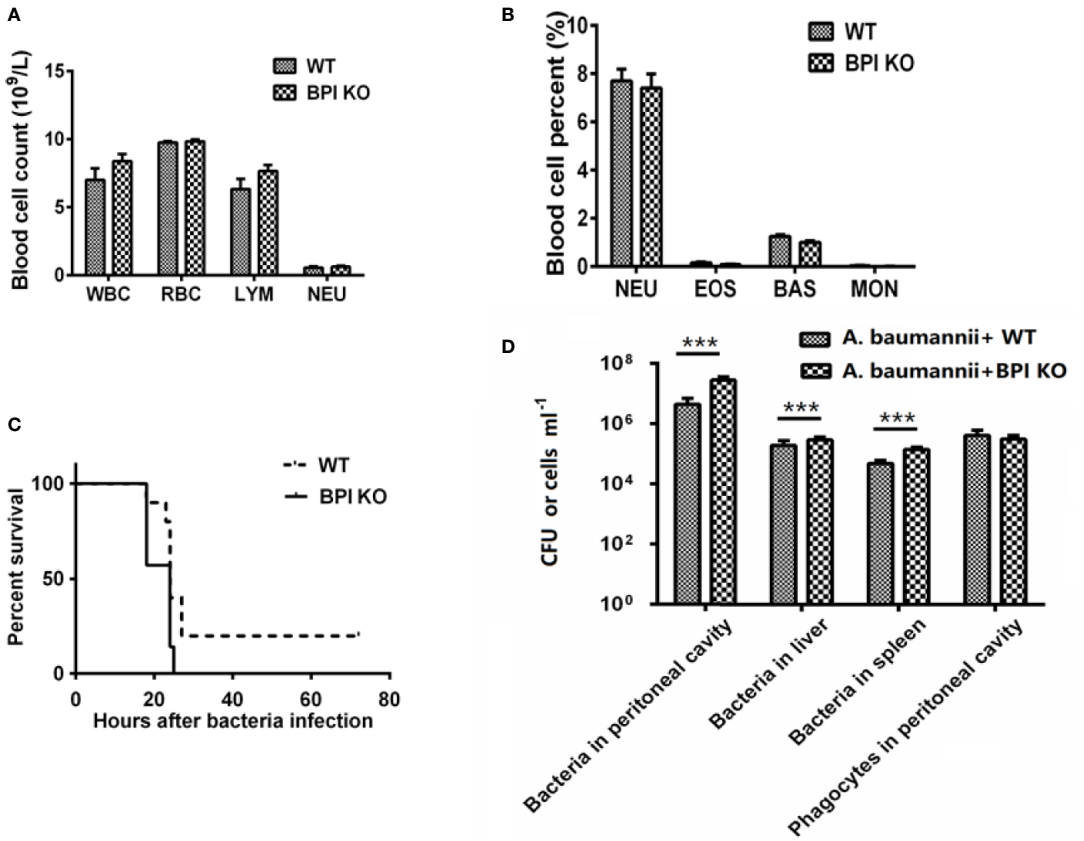

FIGURE 3 | Survival of mice after intraperitoneal bacterial challenge. **(A, B)** the mice were used to count blood cells, the above values were mean ± standard deviation, BPI KO mice (n = 4), WT mice (n = 4). The name of abbreviation in the figure **(A, B)**: White blood cells (WBC), Red blood cells (RBC), Lymphocytes (LYM), Neutrophils (NEU), Eosinophils (EOS), Basophils (BAS), Monocytes (MON). **(C)** Survival of mice 72 h after an i.p. injection of *Acinetobacter baumannii* (0.5×10^4 CFU) in 5% inactivated yeast solution. BPI KO mice (n = 10), WT mice (n = 10). **(D)** The number of bacteria and peritoneal phagocytes in the mice after intraperitoneal challenge with *A. baumannii*. BPI KO mice (n = 5), WT mice (n = 5), ***p < 0.001. All experiments were repeated two times independently.

significantly higher than those in the WT mice (1.825 ± 0.04794, n = 3) (P = 0.0146) (**Figure 6B**). These results suggest that loss of BPI results in an increase of the epithelial permeability in colon, which might cause LPS release from lumen of colon into blood.

Increased Lipopolysaccharide in the Serum of Bactericidal Permeability Increasing Protein Knockout Mice Leads to Aggravation of Dextran Sulfate Sodium-Induced Mouse Colitis

On the 5th day of 5% DSS drinking, we did not detect any bacteria in the anti-coagulated blood and splenic homogenates collected from the mice (data not provided). Therefore, we measured the LPS levels in the serum of the mice. The serum LPS levels in the BPI KO mice were significantly higher than those in the WT mice (P =0.0159) (**Figure 7A**). Five days after the mice started drinking 5% DSS, the serum LBP levels in the BPI KO mice (n = 5) were higher than those in the WT mice (n = 5), but the difference was not significant (**Figure 7B**). Our results suggest that loss of BPI can increase the levels of LPS in the blood.

A cytokine assay using the colonic tissue of mice subjected to 5% DSS for 5 days revealed that the levels of TNF-α, IL-1β, IL-2, IL-3, and IFN-γ were higher in BPI KO mice than in WT mice, while the levels of IL-6, GM-CSF, and MCP-1 were lower than those of the BPI KO mice (**Figure 7C**). Only the difference in the TNF-α levels was significant between the BPI KO mice and WT mice (P = 0.0267).

MPO detection in mouse colon tissue found that BPI KO mice had higher MPO values than WT mice on the 5th day of 5% DSS drinking, but the difference was not significant (**Figure 7D**).

16S rDNA Analysis in Mouse Stool Samples

We collected feces from the BPI KO mice to assess microbial differences by high-throughput 16S ribosomal DNA gene sequencing. The Shannon rarefaction plot demonstrated that

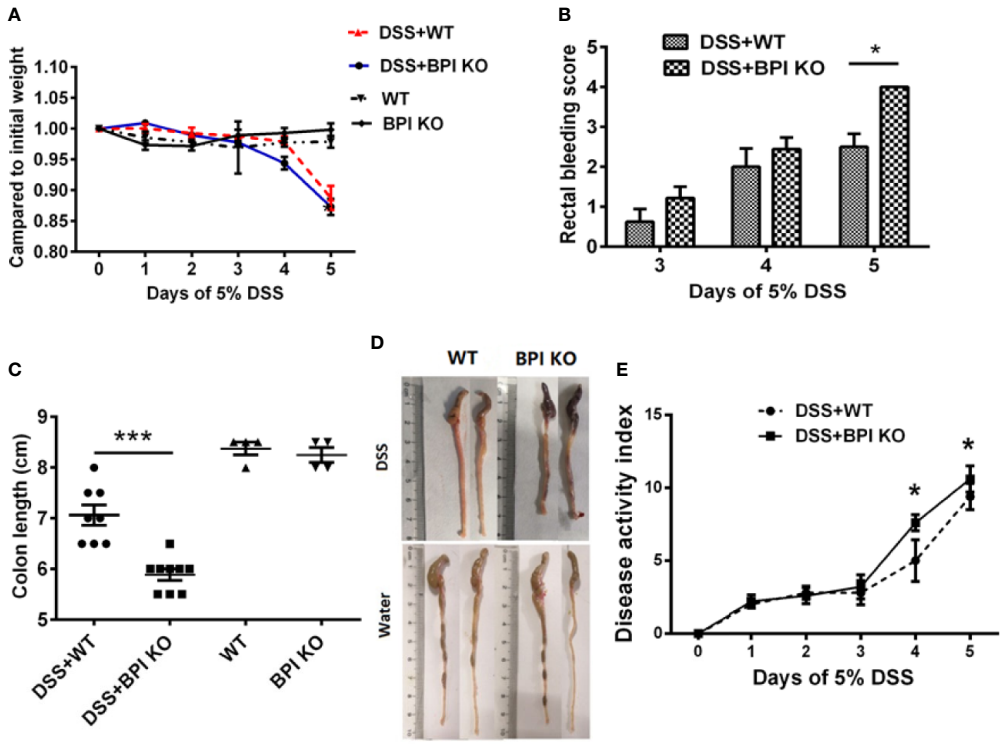

FIGURE 4 | BPI deficiency aggravates colitis symptoms of DSS-induced murine colitis. WT mice (n = 5–8) and BPI KO mice (n = 5–9) were subjected to 5% DSS for 5 days. WT mice (n = 4) and BPI KO mice (n = 4) continuously drank water for 5 days as control. On the 5th day, all mice were sacrificed. **(A)** During the 5-day observation period, changes in body weight were recorded and are expressed as a percentage of the body weight at the start of the experiment. **(B)** Bloody stool scores, $^{*}p < 0.05$. **(C)** Length of the large intestine, $^{***}p < 0.001$. **(D)** General morphology of proximal end of colon on the 5th day after DSS induction. **(E)** Disease activity index (DAI), $^{*}p < 0.05$, $^{***}p < 0.001$. All experiments were performed three times.

the microbial diversity of the feces from KO (BPI KO) mice was lower than that of the WT mice. The Shannon rarefaction plot further demonstrated that the microbial diversity of the feces from DSS_KO (DSS+ BPI KO) mice was lower than that of the DSS_WT (DSS+WT) mice (**Figure 8A**). It was demonstrated that BPI knockout could decreased the microbial diversity of the feces. The heatmap of the genera shows the differences between the various species of flora in the DSS_WT and DSS_KO mice. The greatest differences in the number of bacteria between the DSS_KO and the DSS_WT groups were in unclassified ($P < 0.0001$, $X^2 = 68951$, n = 3), *Bacteroides* ($P < 0.0001$, $X^2 = 14750$, n = 3), and *Lactobacillus* ($P < 0.0001$, $X^2 = 5177$, n = 3) (**Figures 8B, C**). Taken together, these results suggest that a deficiency in BPI regulate the fecal flora in the gut.

DISCUSSION

BPI plays an important role in innate immunity against infection. The inability to produce BPI in newborns is the reason for their susceptibility to gram-negative infections (24). Human BPI is mainly expressed in neutrophils, where it has a bactericidal function, (25) and it has also been demonstrated in neutrophil extracellular traps (26). In this study, we generated BPI global knockout mice, and challenged mice with *A. baumannii*, a gram-negative bacterium (23). Our results showed that the number of *A. baumannii* in the peritoneal, liver and spleen was increased in BPI KO mice. It may be the lost the bactericidal function of BPI in BPI KO mice. Our previous studies showed that the serum LPS levels in mice challenged with *E. coli O111B4*, a gram-negative bacterium increased as the number of bacteria in the liver and spleen increased (27).

BPI is expressed in intestinal epithelial cells, which is the first barrier against infection. Human gastrointestinal epithelial cells also express BPI (6). Both BPI floxed mice and BPI KO mice did not spontaneously develop colitis, indicating that BPI deletion within intestinal epithelial cells is not the cause of ulcerative colitis. This may be because the intestinal mucosa relies on a variety of antimicrobial proteins or peptides to maintain its stability (28).

Clinically, human BPI is related to ulcerative colitis (11–15). We used DSS as a model to induce acute experimental colitis in mice, as previously reported (15), in order to understand the role of BPI in the pathogenesis of ulcerative colitis. Our results showed that colonic lesions were found mainly on the mucosal

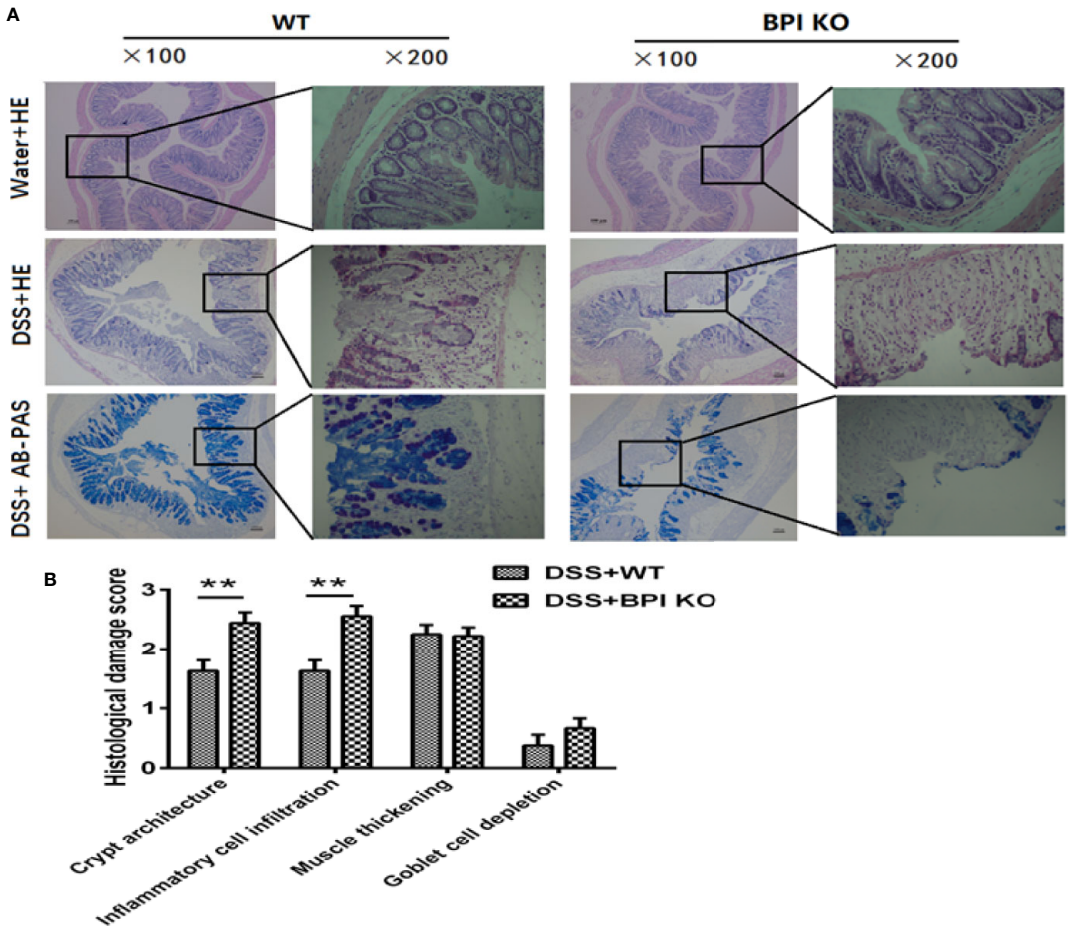

FIGURE 5 | BPI deficiency aggravates colonic mucosal damage of DSS-induced murine colitis. WT mice (n = 5) and BPI KO mice (n = 5) were subjected to 5% DSS for 5 days. WT mice (n = 4) and BPI KO mice (n = 4) continuously drank water for 5 days as control. On the 5th day, all mice were sacrificed. **(A)** Colonic images after H&E staining and AB-PAS staining (original magnification x 100 and x 200). The difference between the two groups was mainly within the colonic mucosa, such as severe crypt distortion, inflammatory cell infiltration and globlet cell depletion. **(B)** Colonic pathological scores, $^{**}p < 0.01$. All experiments were performed three times.

surface, indicating that knockout of BPI promoted more severe damage to the colonic mucosa of the mice, which was consistent with the clinical hypothesis that BPI plays a protective role in the early stages of ulcerative colitis (12).

DSS is toxic to intestinal epithelial cells, which causes a decrease in the mucosal barrier, resulting in increased bacterial translocation. This causes blood neutrophils to infiltrate into the local infection site by chemotaxis *via* IL-8. These cells maintain the intestinal stability by phagocytosing and killing bacteria. Our results showed that BPI was expressed at sidewall and bottom of colon crypts in WT mice. In colitis model, the level of BPI was increased at bottom of colon crypts but decreased at the sidewall of crypt which may due to the mucosal injury. A BPI deficiency resulted in an increase of epithelial permeability of colon which led to LPS from colon into blood. This increased serum LPS stimulated the liver to synthesize and secrete increased levels of serum LBP. LPS permeabilized from colon also can induced an increase in the levels of various cytokines, such as TNF-α, IL-1β, IL-2, IL-3, and IFN-γ in the gut. The MPO results showed no significant changes between WT and KO mice, indicating that BPI deficiency might not affect neutrophils, which is the major resource of MPO. As reported before (29), BPI protein is closely associated with dendritic cell, and dendritic cell is a bridge between innate and adaptive immunity, which need to study in the future.

We did not detect any bacteria in the anti-coagulated blood and spleen homogenates of mice subjected to DSS. We then assessed the fecal flora of the mice. Our results showed that BPI knockout decreased the fecal flora diversity. The greatest differences in the number of bacteria were in unclassified and gram-negative bacteria, such as *Bacteroides.*

In summary, our study constructed BPI KO mice, which were then subjected to dextran sulfate sodium (DSS) colitis. The results showed that BPI KO developed worse colitis than

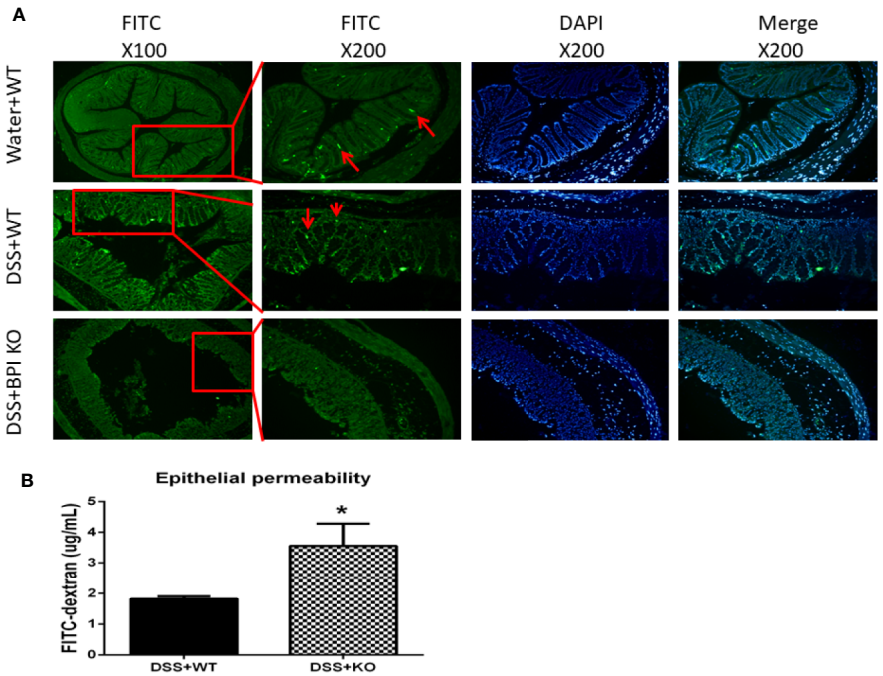

FIGURE 6 | BPI KO leads to an increase of epithelial permeability of colon of DSS-induced mouse colitis. WT mice (n = 3) and BPI KO mice (n = 3) were subjected to 5% DSS for 5 days. WT mice (n = 3) continuously drank water for 5 days as control. **(A)** Under the fluorescence microscope, WT mice expressed BPI dominantly at sidewall and bottom of crypt of colon visualized by FITC fluorescence (green). In colitis model, WT mice expressed BPI dominantly at bottom of crypt and BPI deficiency was at colon of BPI KO mice. **(B)** Plasma concentration of fluorescein was measured at 4h after gavage FITC–dextran, $^{*}p < 0.05$. All experiments were performed two times.

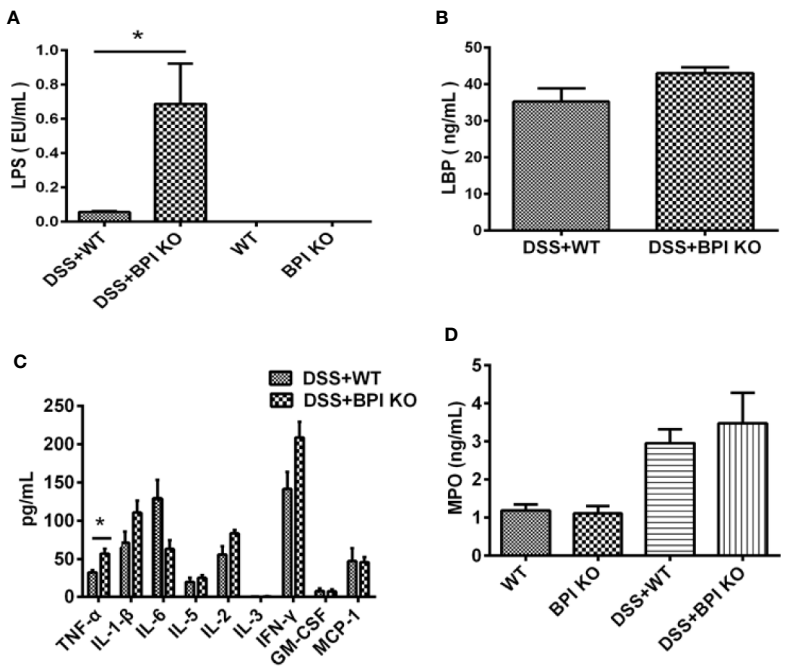

FIGURE 7 | BPI deficiency increases serum LPS levels of DSS-induced murine colitis. WT mice (n = 5) and BPI KO mice (n = 5) were subjected to 5% DSS for 5 days. WT mice (n = 4) and BPI KO mice (n = 4) continuously drank water for 5 days as control. On the 5th day, all mice were sacrificed. **(A)** Serum LPS levels s, $^{*}p < 0.05$. **(B)** Serum LBP levels. **(C)** Cytokines levels in proximal end of colon tissues, $^{*}p < 0.05$. **(D)** MPO levels in proximal end of colon tissue. All experiments were performed three times.

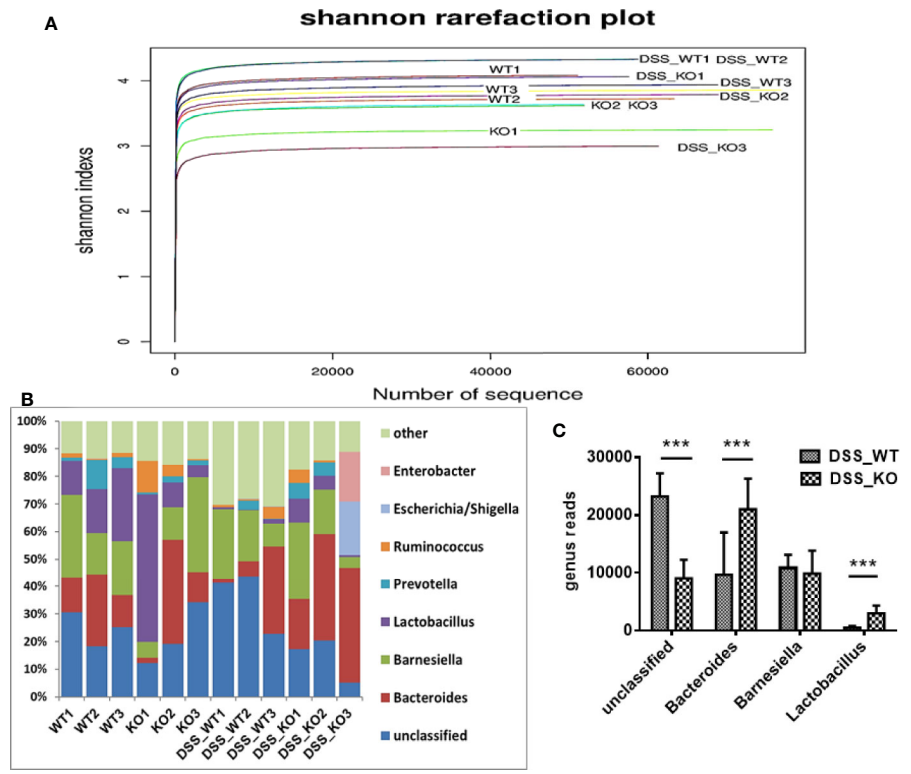

FIGURE 8 | 16S rDNA analysis in mouse stool samples. **(A)** Shannon rarefaction plot. The Shannon rarefaction plot showed that the microbial diversity of the feces from DSS_KO (DSS+ BPI KO) mice was lower than that of the DSS_WT (DSS+WT) mice. It was demonstrated that BPI knockout could decreased the microbial diversity of the feces. **(B, C)** Heatmap of the genera. The number of unclassified, *Bacteroides*, *Barnesiella*, and *Lactobacillus* as compared to the total number of bacteria in the DSS_WT and DSS_KO mice, ***$p < 0.001$. All experiments were performed twice.

control mice, as evidenced by increased colitis symptoms and colonic mucosal damage, elevated levels of serum LPS, and a disrupted microbiome. Thus, BPI may be a potential target for the treatment of ulcerative colitis (UC) in humans.

DATA AVAILABILITY STATEMENT

The 16s rDNA data has been uploaded to NCBI—BioProject ID: PRJNA670293.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Experiment and Experimental Animal Welfare Committee of Capital Medical University.

AUTHOR CONTRIBUTORS

The study was designed by QK, YA, and JZ. Experiments were performed by QK, ZL, YK, and LL. Results were analyzed by QK and ZL. QK wrote the paper. All authors read and revised the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.614169/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | RT-PCR results of BPI KO mouse testis were sequenced and compared with murine BPI mRNA. Using the BLAST function on the NCBI website, the sequencing results were compared with the mouse BPI mRNA. The 200 bp fragment (Query) overlaps with the tail of the 1st exon (1–184) and the head of the 4th exon (429–587), but the 2nd and 3rd exons were absent. Thus, the 2nd and 3rd exons of BPI were successfully knocked out of the mice.

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ORIGINAL RESEARCH
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Anethole Attenuates Enterotoxigenic *Escherichia coli*-Induced Intestinal Barrier Disruption and Intestinal Inflammation via Modification of TLR Signaling and Intestinal Microbiota

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This study aimed to investigate the effects of dietary anethole supplementation on the growth performance, intestinal barrier function, inflammatory response, and intestinal microbiota of piglets challenged with enterotoxigenic *Escherichia coli* K88. Thirty-six weaned piglets (24 ± 1 days old) were randomly allocated into four treatment groups: (1) sham challenge (CON); (2) *Escherichia coli* K88 challenge (ETEC); (3) *Escherichia coli* K88 challenge + antibiotics (ATB); and (4) *Escherichia coli* K88 challenge + anethole (AN). On day 12, the piglets in the ETEC, ATB, and AN group were challenged with 10 mL *E. coli* K88 (5×10^9 CFU/mL), whereas the piglets in the CON group were orally injected with 10 mL nutrient broth. On day 19, all the piglets were euthanized for sample collection. The results showed that the feed conversion ratio (FCR) was increased in the *Escherichia coli* K88-challenged piglets, which was reversed by the administration of antibiotics or anethole ($P < 0.05$). The duodenum and jejunum of the piglets in ETEC group exhibited greater villous atrophy and intestinal morphology disruption than those of the piglets in CON, ATB, and AN groups ($P < 0.05$). Administration of anethole protected intestinal barrier function and upregulated mucosal layer (mRNA expression of mucin-1 in the jejunum) and tight junction proteins (protein abundance of ZO-1 and Claudin-1 in the ileum) of the piglets challenged with *Escherichia coli* K88 ($P < 0.05$). In addition, administration of antibiotics or anethole numerically reduced the plasma concentrations of IL-1β and TNF-α ($P < 0.1$) and decreased the mRNA expression of TLR5, TLR9, MyD88, IL-1β, TNF-α, IL-6, and IL-10 in the jejunum of the piglets after challenge with *Escherichia coli* K88 ($P < 0.05$). Dietary anethole supplementation enriched the abundance of beneficial flora in the intestines of the piglets. In summary, anethole can improve the growth performance of weaned piglets infected by ETEC through attenuating intestinal barrier disruption and intestinal inflammation.

Keywords: anethole, piglet, *Escherichia coli* K88, antibiotics, microbiota

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INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is considered one of the main causes of diarrhea in weaning piglets (Fairbrother et al., 2005). Generally, a poor breeding environment causes an increase in intestinal ETEC (Yokoyama et al., 1992), disrupts the balance of intestinal flora (Li et al., 2012) and affects the digestion and absorption of nutrients (Gao et al., 2013). The enterotoxins secreted by ETEC can destroy the intestinal mucosa layer and tight junction structure, which leads to increased permeability of the intestine (Fleckenstein et al., 2010; Dubreuil, 2012). Bacteria or antigens that pass through the intestinal mucosa are captured and recognized by immune cells, which further activate the immune response and inflammatory process (Moretó and Pérez-Bosque, 2009; Turner, 2009). Damage to the intestine could reduce growth performance, cause severe diarrhea, and even lead to piglet death (Fleckenstein et al., 2010).

A large number of antibiotics are used in animal production worldwide each year, of which most are antibiotic growth promoters (AGPs). Based on a survey of antibiotic usage in China for 2013, total antibiotic production usage was approximately 162,000 tons, of which 52% was used in animals (Ying et al., 2017). According to the requirements of Chinese government, feed manufacturers is not allowed to produce commercial feeds containing growth-promoting drug feed additives since July 1, 2020.

"Medicine and food are homologous" comes from a view of traditional Chinese medicine, meaning that some of food can have a certain therapeutic effect. Anethole (AN) was originally extracted from fennel and has long been proven to have anti-inflammatory effects, and that it's also has been used in animal production (Windisch et al., 2008). Previous studies have reported that AN can improve the growth performance of animals at an appropriate dosage (Kim et al., 2013; Zeng et al., 2015; Charal et al., 2016). However, to the best of our knowledge, there is no comprehensive report on the effects of AN on ETEC-infected piglets. Thus, the primary aim of this study was to determine the effects of AN on the growth performance, intestinal barrier function, inflammatory response and intestinal microbiota of piglets challenged with ETEC.

MATERIALS AND METHODS

Ethics Approval

All the experimental protocols in this study were satisfy the needs of animal welfare and conducted in strict accordance with the Guidelines for the Protection and Use of Laboratory Animals issued by the South China Agricultural University Animal Care and Use Committee (No. 20110107-1, Guangzhou, China).

Animals, Housing, and Experimental Design

This trial is conducted in an experimental house with a controlled temperature at 30 ± 2°C and humidity below 80%. Piglets were individually fed in metabolic cages (1.2 m × 0.4 m × 0.5 m) with a three-day adaptation period to adapt to the new environment and feed. All piglets had free access to feed and water. During the adaptation period, piglets did not show any symptoms of diarrhea, skin lesions and obvious inflammation, which indicated that piglets were healthy and suitable for this experiment. After adaption, 36 male piglets (Duroc × (Landrace × Yorkshire), initial weight 7.5 ± 1 kg) were assigned to one of four treatments according to the principle of similar weight (n = 9). This experiment last for 19 days. Four treatments are listed as follows: (1) sham challenge (CON); (2) *Escherichia coli* K88 challenge (ETEC); (3) *Escherichia coli* K88 challenge + antibiotics (ATB); and (4) *Escherichia coli* K88 challenge + anethole (AN). CON and ETEC groups receives the control diet, ATB group receives the control diet supplemented with antibiotics (50 mg/kg quinocetone, 75 mg/kg chlortetracycline, 50 mg/kg kitasamycin), and AN group receives the control diet supplemented with AN (300 mg/kg, pure AN ≧ 7.5%, coating with corn starch, Pancosma, Switzerland). The feed formula was prepared according to NRC (2012). The ingredient composition and nutrient levels of control diet are presented in **Supplementary Table 1**.

Enterotoxigenic *Escherichia coli* K88 Challenge

Escherichia coli K88 (CVCC225) was purchased from the Chinese Veterinary Medicine Collection Center, and it was confirmed to have heat labile enterotoxin (LT), heat stable enterotoxin (ST), and F4 fimbriae in our laboratory (Ren et al., 2019). On day 12, piglets from the ETEC, ATB, and AN group orally challenged with 10 mL nutrient broth (NB) containing 5 × 10^9 CFU/mL ETEC K88 via a syringe, CON group orally injected with 10 mL of sterilized NB. CON group was kept in isolation in order to avoid cross-contamination.

Sample Collection

Blood and feces samples were collected on day 19. Five milliliter of blood samples were collected into tubes containing EDTA via the anterior vena cava puncture and quickly centrifuged (1000 × g, 4°C, 10 min) for plasma samples in 30 min, then stored at −80°C. At the same time, over 5 g fresh feces samples were collected into centrifuge tubes and stored at −80°C. After blood and feces samples collection, piglets were immediately euthanized. About 2 cm length of duodenum (about 10 cm near the pylorus) and jejunum (about 60 cm near the pylorus) were collected, then stored into 4% paraformaldehyde solution for histological analyses. Jejunal and ileal segments (10 cm length) were opened longitudinally and the contents were flushed in cold

Abbreviations: ADFI, average daily feed intake; ADG, average daily gain; AN, anethole; ATB, antibiotics; BW, body weight; CON, control; ETEC, enterotoxigenic *Escherichia coli*; F/G, feed conversion ratio; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-10, interleukin-10; LPS, lipopolysaccharide; MLN, mesenteric lymph nodes; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor kappa B; NMDS, Non-metric multi-dimensional scaling; OTU, operational taxonomic unit; PCoA, principal coordinate analysis; SEM, standard error of mean; SIGIRR, single immunoglobulin and toll-interleukin 1 receptor domain; TLR, toll like receptor; TNF-α, tumor necrosis factor-α; TOLLIP, toll interacting protein; TRAF6, tumor necrosis factor receptor-associated factor 6; VCR, villi height and crypt depth ratio; ZO-1, Zonula occludens protein-1.

normal saline (NS) solution for twice. Mucosa was collected by scraping using a sterile glass microscope slide at 4°C, rapidly frozen in liquid N_2 and stored at −80°C for the analysis of mRNA and protein expression. Similarly, mesenteric lymph node (MLN) was collected and rapidly frozen in liquid N_2 for the analysis of mRNA expression. The time from anesthesia to complete sampling was controlled at about 30 min per piglet.

Measurements

Growth Performance

Feed intake of each piglets was daily recorded. Body weight of each piglets were recorded on day 0, day 12, and day 19 to calculate average daily gain (ADG), average daily feed intake (ADFI) and F/G (Feed conversion ratio) respectively.

Immunological Parameters

Plasma IL-1β, TNF-α, IL-6, and IL-10 were analyzed by using commercially available porcine ELISA kits (Huamei, Wuhan), according to the manufacturer's instructions. All assays were run in duplicate and diluted if necessary.

Intestinal Morphology

The samples of duodenum and jejunum were embedded in paraffin. Each sample was used to prepare one slide with two sections (4 μm thickness), which were stained with Hematoxylin-Eosin. Three views of each section and three well-oriented villi and crypts of each view were selected for intestinal morphology measurement. Villi height and crypt depth ratio (VCR) was calculated after measure.

Quantitative PCR for Gene Expressions

Total RNA was extracted from the frozen jejunum, ileum, and MLN tissues by using total RNA extraction kit (LS040, Promega, Shanghai, China) according to the manufacturer's instruction. The quality, purity and concentrations of RNA samples were assessed by electrophoresis on 1.5% agarose gel (130 V, 18 min) and NanoDrop Spectrophotometer (A260/A280). Then, the RNA was adjusted to a uniform concentration by using RNase-free ddH_2O. Subsequently, reverse transcription of the RNA to complementary cDNA was performed using a cDNA reverse transcription kit (RR047A, Takara, Tianjin, China). Quantitative PCR by using the SYBR green system (RR820A, Takara) was performed on QuantStudioTM 6 Flex (Applied Biosystems, CA, United States). The reaction mixture (10 μL) contained 5 μL of SYBR Green PCR Master Mix, 1 μL of cDNA, 0.4 μL of forward and reverse primer (10 μM/L), 0.2 μL of ROX Reference Dye II (50×), and 3 μL of RNase-free ddH_2O. The PCR reaction was repeated three times for each gene and carried out as following: one cycle at 50°C for 120 s and 95°C for 600 s, forty cycles at 95°C for 15 s and 60°C for 60 s and one cycle at 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s. Target gene expression was calculated based on the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and normalized to GAPDH. The primer sequences were designed by using Primer 3.0 (**Supplementary Table 2**).

Western Blot Analysis for Protein Expressions

The total protein in the frozen jejunal and ileal tissue samples was lysed in RIPA (P0013B, Biyuntian, Shanghai, China). The protein concentration of each sample was measured using BCA protein assays (P0010, Biyuntian, Shanghai, China). Equal amounts of denatured protein (25 μg) from each sample were separated on 10% SDS-PAGE and then electroblotted onto PVDF membranes. Membranes were blocked for 2 h with 5% skimmed milk in TBST at room temperature. Subsequently, the membranes were incubated with specific antibody [ZO-1 (ab96b87, Abcam, United States), Occludin (ab31721, Abcam, United States), Claudin-1 (ab15098, Abcam, United States), and β-actin (bs-0061R, Bioss, China)] for 12 h at 4°C, and then incubate with secondary antibody for 1 h at room temperature. Finally, the proteins were detected using ECL chemiluminescence reagents (P1020, ApplyGen, Beijing, China) and FluorChem M Fluorescent Imaging System (ProteinSimple, CA, United States). Protein expression were analyzed by using image J software.

16S rDNA Sequencing

Gut microbiome of feces was analyzed by High-throughput 16S rDNA sequencing technology. Sequencing (PE250, NovaSeq6000, Illumina Inc., CA, United States) was performed by Novogene Co., Ltd. (Beijing, China), the V3–V4 region of the 16S rDNA was amplified using primers 341F (5′-CCTAYGGGRBGCASCAG) and 806R (5′-GGACTACNNGGGTATCTAAT). For 16S rDNA sequencing data, statistical analyses were performed with NovoMagic (Novogene Co., Ltd.) online tools.

Statistical Analysis

All data of this experiment were analyzed by using one-way ANOVA according to the general linear models (GLM) procedure of SPSS 22.0 (IBM Inc., United States). Data were expressed as means ± SEM. Comparisons between the values of mean treatment were made by LSD using Duncan's multiple test. $P < 0.05$ was considered as statistically significant differences and as tendency when $0.05 < P < 0.10$.

TABLE 1 | Effects of anethole on growth performance of piglets challenged with enterotoxigenic *Escherichia coli* K88.

Items	Treatments				SEM	*P*-value
	CON	ETEC	ATB	AN		
Initial BW (kg)	7.17	7.13	7.32	7.25	0.16	0.977
Final BW (kg)	13.78	12.69	13.60	13.51	0.41	0.795
Pre-challenge (0–12 days)						
ADFI (g)	357	358	355	371	18	0.990
ADG (g)	275	272	280	285	14	0.989
F/G	1.33	1.30	1.31	1.33	0.04	0.995
Post-challenge (13–19 days)						
ADFI (g)	623	559	623	608	27	0.832
ADG (g)	445	307	406	394	22	0.136
F/G	1.47^b	1.87^a	1.52^b	1.55^b	0.06	0.050

n = 9. Different letters mean statistically significant difference among the groups (P < 0.05).

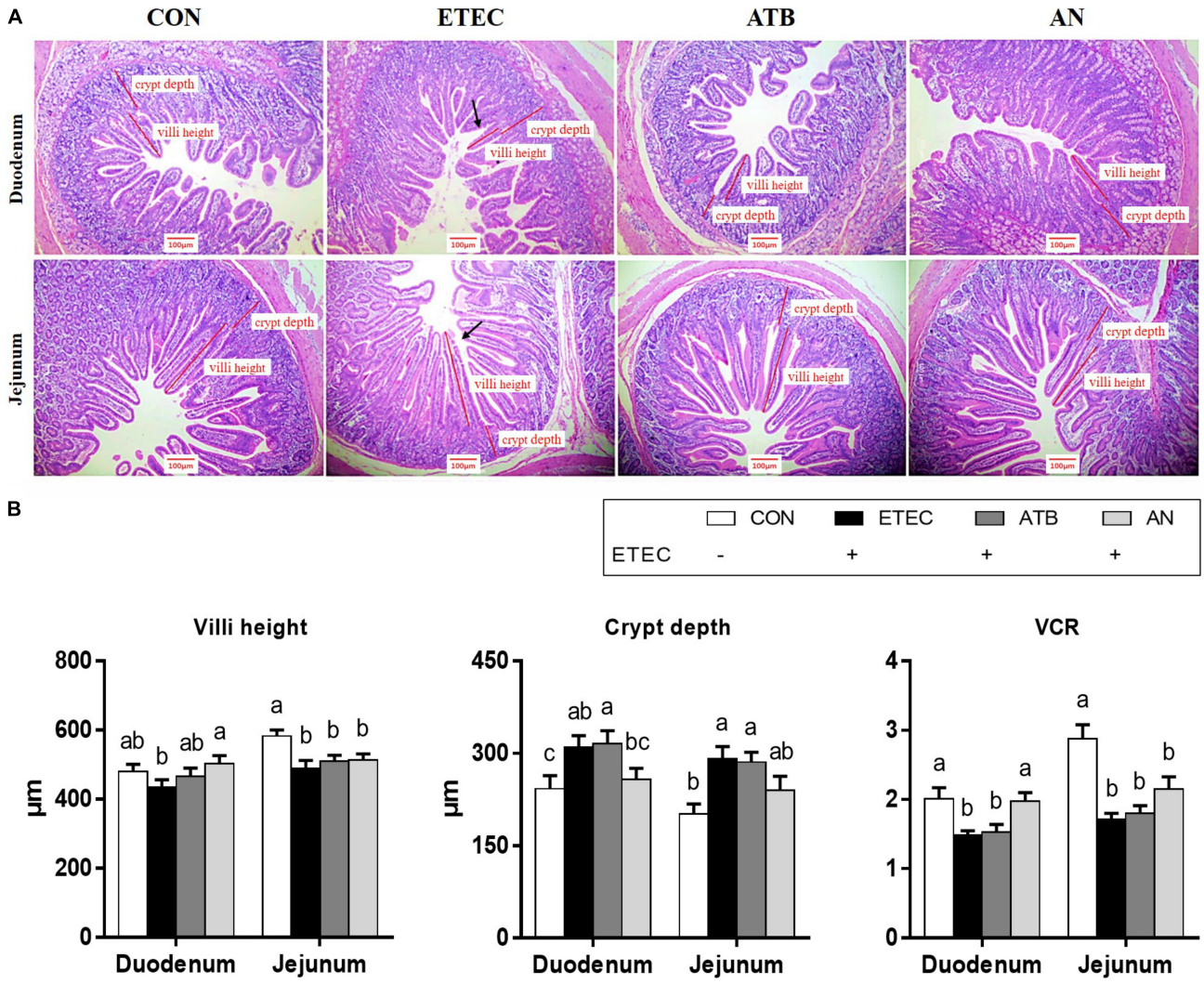

FIGURE 1 | Effects of anethole on intestinal morphology of piglets challenged with enterotoxigenic *Escherichia coli* K88. **(A)** HE staining of intestine **(B)** Villi height, crypt depth and VCR of intestine. The data in each group is expressed as the mean ± SE (n = 9). Different letters mean statistically significant difference among the groups ($P < 0.05$).

RESULTS

Performance

After ETEC challenge, the piglets fed diets containing antibiotics or AN had lower ($P < 0.05$) F/G than the piglets challenged with ETEC, and this F/G was similar to that of the piglets given the CON treatment (**Table 1**).

Intestinal Morphology

The duodenums of the piglets in the AN group exhibited higher villus heights ($P < 0.05$) and VCRs ($P < 0.05$) than those of the piglets in the ETEC group. The piglets in the ATB group had greater duodenal crypt depths ($P < 0.05$) than those in the CON and AN groups. The jejunums of the piglets in the CON group had higher villus heights ($P < 0.05$) and VCRs (**Figure 1**, $P < 0.05$) than those of the piglets in the other groups. In addition, the piglets in the CON group had lower crypt depths (**Figure 1**, $P < 0.05$) than those in the ETEC and ATB groups.

Barrier Function

The relative mRNA expression of mucin-1, mucin-2, ZO-1, Occludin, and Claudin-1 in the jejunum and ileum of the piglets was significantly downregulated ($P < 0.05$, **Figure 2**) in response to ETEC challenge ($P_{mucin-2} = 0.063$). However, this downregulation was partially mitigated by dietary supplementation with antibiotics or AN. In addition, the ZO-1, Occludin, and Claudin-1 protein levels were also markedly decreased ($P < 0.05$, **Figure 2**) after ETEC challenge, which can be attenuated by dietary supplementation with antibiotics or AN.

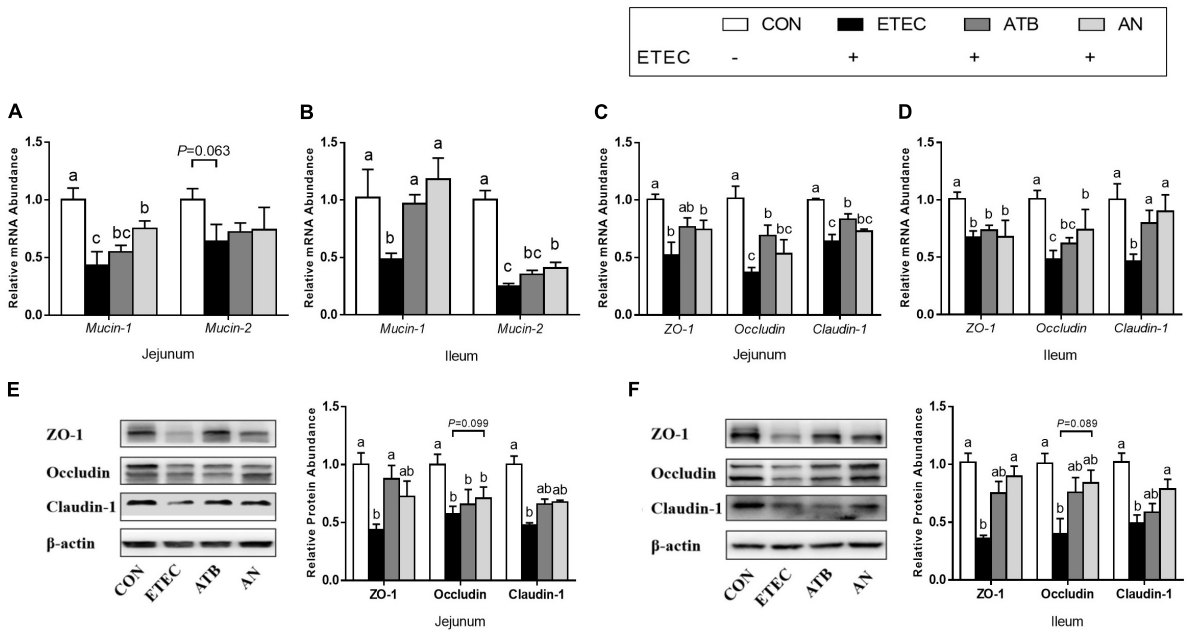

FIGURE 2 | Effects of anethole on **(A–B)** intestinal secretory and **(C–F)** barrier function of piglets challenged with enterotoxigenic *Escherichia coli* K88. For PCR assays, $n = 8$, *GAPDH* as reference gene; For western blot, $n = 3$, β-actin as reference protein; The data in each group is expressed as the mean ± SE. Different letters mean statistically significant difference among the groups ($P < 0.05$).

Inflammatory and Immunological Responses

The plasma levels of IL-1β and TNF-α in the piglets tended to increase after ETEC challenge ($P < 0.1$), whereas this tendency was not observed in the ETEC-challenged piglets in the ATB and AN groups. Compared with those of the piglets in the CON group, the relative mRNA expression levels of certain genes were upregulated ($P < 0.05$) in the jejunum (TLR9, MyD88 and IL-10), ileum (TLR5), and MLN (NF-κB) of the piglets in the ETEC group, which could be attenuated by supplementation with antibiotics and AN. Compared with those of the piglets in the ATB group, the relative mRNA expression levels of certain genes was significantly decreased ($P < 0.05$) in the jejunum (TLR5 and TLR9) and significantly increased ($P < 0.05$) in the MLN (TRAF6) of the piglets in the AN group. In addition, compared with those of the piglets in the CON or ETEC group, the relative mRNA expression levels of genes related to the MyD88/NF-κB signaling pathway were upregulated ($P < 0.05$) in the jejunum (SIGIRR) and ileum (SIGIRR and IL-10) of the piglets in the ETEC group and the ileum (SIGIRR) and MLN (SIGIRR) of the piglets in the AN group (**Figure 3**).

Gut Microbiome

A total of 1,775,153 high-quality sequences were generated from 20 fecal samples (four treatments, $n = 5$), with an average of 88,758 sequences per sample, and 64,854 ± 2,566 effective tags were obtained for subsequent analysis after the noise sequences were discarded. Finally, all the effective tags were clustered to operational taxonomic units (OTUs) at 97% sequence similarity and then allotted to 23 phyla, 39 classes, 81 orders, 145 families, 322 genera, and 1,738 OTUs. For alpha-diversity, the bacterial richness ACE and Chao1 index of the AN group were markedly higher than those of the CON and ETEC groups ($P < 0.05$), the richness Observed_species index of the ETEC and AN group had a tendency of significant difference ($0.05 < P < 0.10$), the diversity Shannon and the Simpson index had no significant difference among four treatment groups ($P > 0.05$). For beta diversity, the PCoA (PC1 32.33% vs PC2 20.26%) and NMDS (stress = 0.133) analyses based on Weighted UniFrac distances showed that the microbiota from the piglets in the ETEC group obviously tended to separate from that of the piglets in both the ATB and AN group (**Figure 4**).

At the phylum level, five major bacteria in the feces of piglets were Firmicutes (51.74–9.85%), Bacteroidetes (6.97–34.30%), Spirochaetes (0.35–13.06%), Actinobacteria (0.84–7.26%), and Euryarchaeota (0.01–5.69%). At the genus level, unidentified_*Clostridiales* (1.59–35.52%), *Catenibacterium* (0.30–16.21%), *Blautia* (0.81–15.16%), *Lactobacillus* (0.51–13.55%), *Terrisporobacter* (0.37–12.64%), and *Catenisphaera* (0.20–10.97%) were the most predominant genera in all the samples, and three genera (*Lactobacillus*, unidentified_*Ruminococcaceae*, and *Selenomonas*) were significantly different among the different groups on top 10 (**Figure 5** and **Supplementary Table 4**). *Lactobacillus* abundance in the ATB group was significantly higher ($P < 0.05$) than that in the ETEC group. unidentified_*Ruminococcaceae* abundance in the ATB and AN groups was significantly lower ($P < 0.05$) than that in the ETEC group. *Selenomonas* abundance in the AN group was significantly higher ($P < 0.05$) than that in the ETEC group.

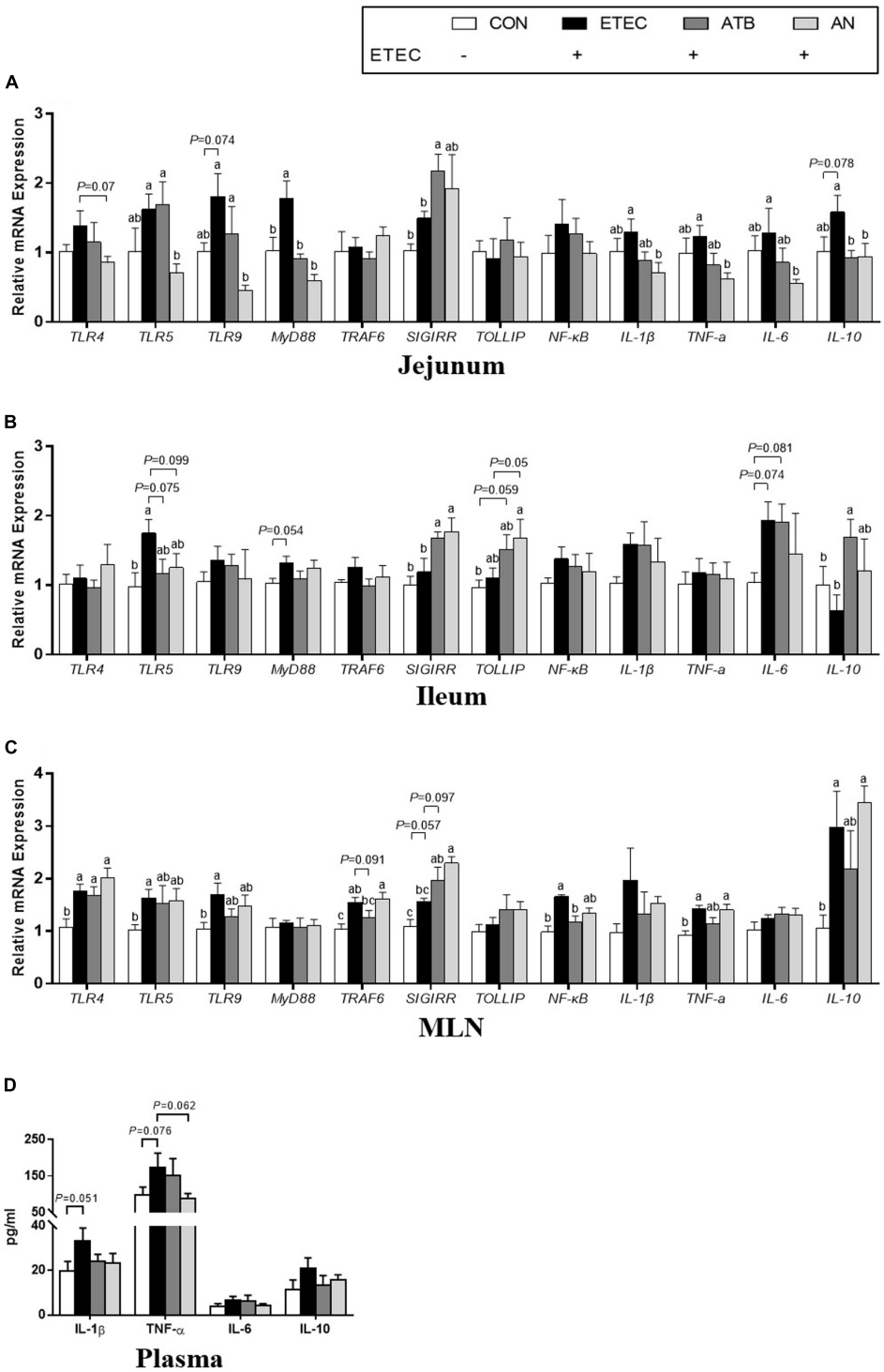

FIGURE 3 | Effects of anethole on concentration of relative mRNA expression of genes related to inflammation **(A)** jejunum, **(B)** ileum, **(C)** MLN, and **(D)** plasma cytokine and of piglets challenged with enterotoxigenic *Escherichia coli* K88. ELISA for plasma, $n = 9$; For PCR assays, $n = 8$, *GAPDH* as reference gene; The data in each group is expressed as the mean ± SE. Different letters mean statistically significant difference among the groups ($P < 0.05$).

DISCUSSION

Enterotoxigenic *Escherichia coli* regulates the secretion of enterotoxins and induces diarrhea and intestinal impairment in weaned piglets (Che et al., 2017). In the present study, the FCR of piglets was significantly increased after ETEC challenge. Correspondingly, after statistical analysis of the internal morphology of piglets, we can find that after ETEC

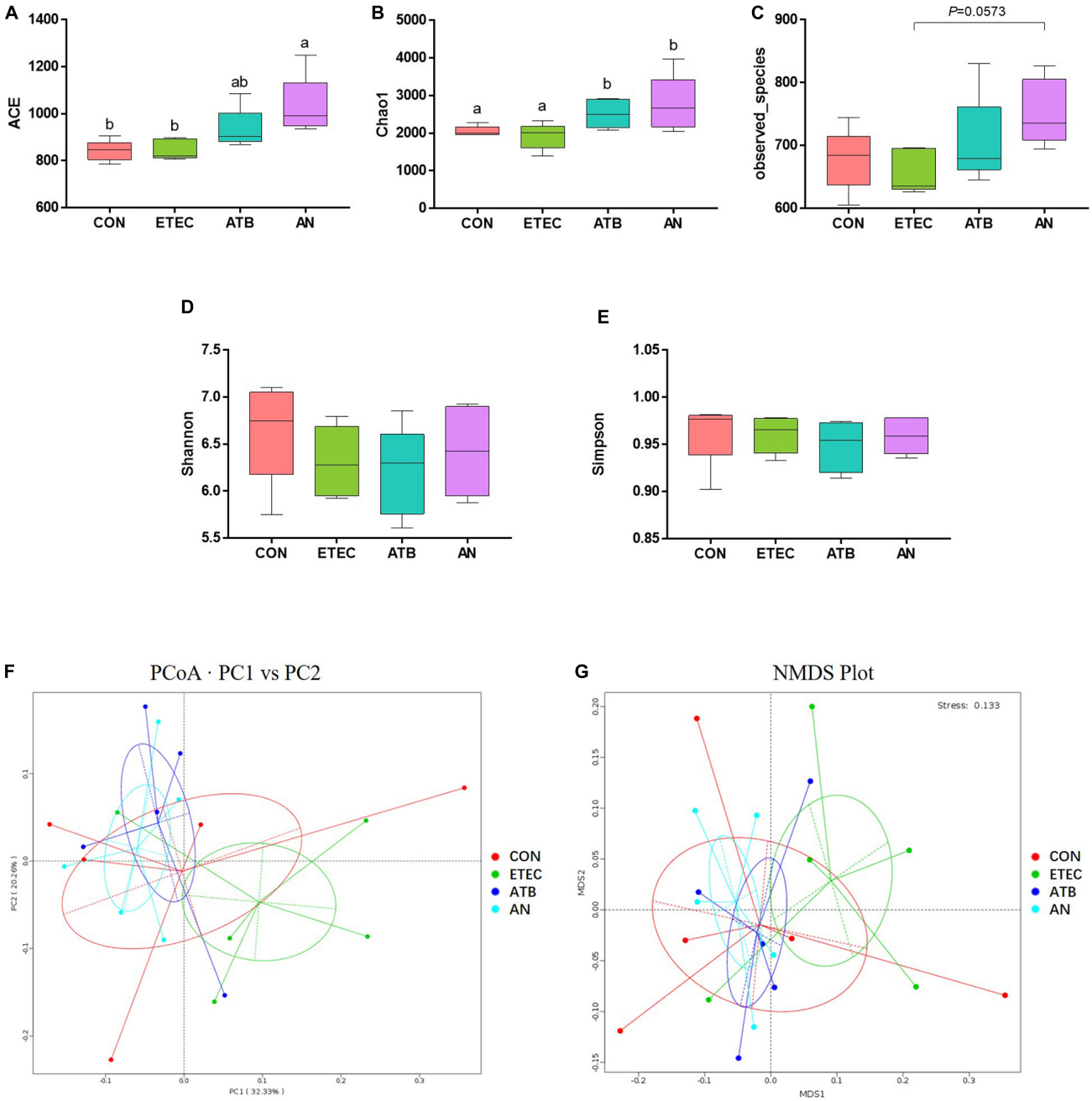

FIGURE 4 | Effects of anethole on alpha diversity and beta diversity of fecal microbiota of piglets challenged with enterotoxigenic *Escherichia coli* K88 (n = 5). **(A–E)** Alpha Diversity index: ACE, Chao1, Observed_species, Shannon, Simpson. **(F)** Principal Coordinates Analysis based weighted Unifrac. **(G)** NMDS Plot Analysis based weighted Unifrac.

challenge, the VCR values of duodenum and jejunum of piglets in ETEC group were significantly reduced ($P < 0.05$), while the VCR value of duodenum of piglets in AN group did not decrease significantly, which is the most direct evidence that AN can help piglets resist ETEC infection. Maruzzella and Freundlich found that AN has the strong bacteriostatic effect (Maruzzella and Freundlich, 2010). Meanwhile, some other studies have found that AN inhibits the secretion of acetylcholinesterase (AchE) and increases the concentration of acetylcholine (Ach; Bhadra et al., 2011), high Ach levels trigger intestinal smooth muscle contraction and enhance gastrointestinal motility, which may decrease the opportunities for ETEC colonization of the intestine.

Toll-like receptors (TLRs) play an important role in the regulation of innate immunity in animals. Numerous pathogenic molecules have been reported to be recognized by TLRs. For

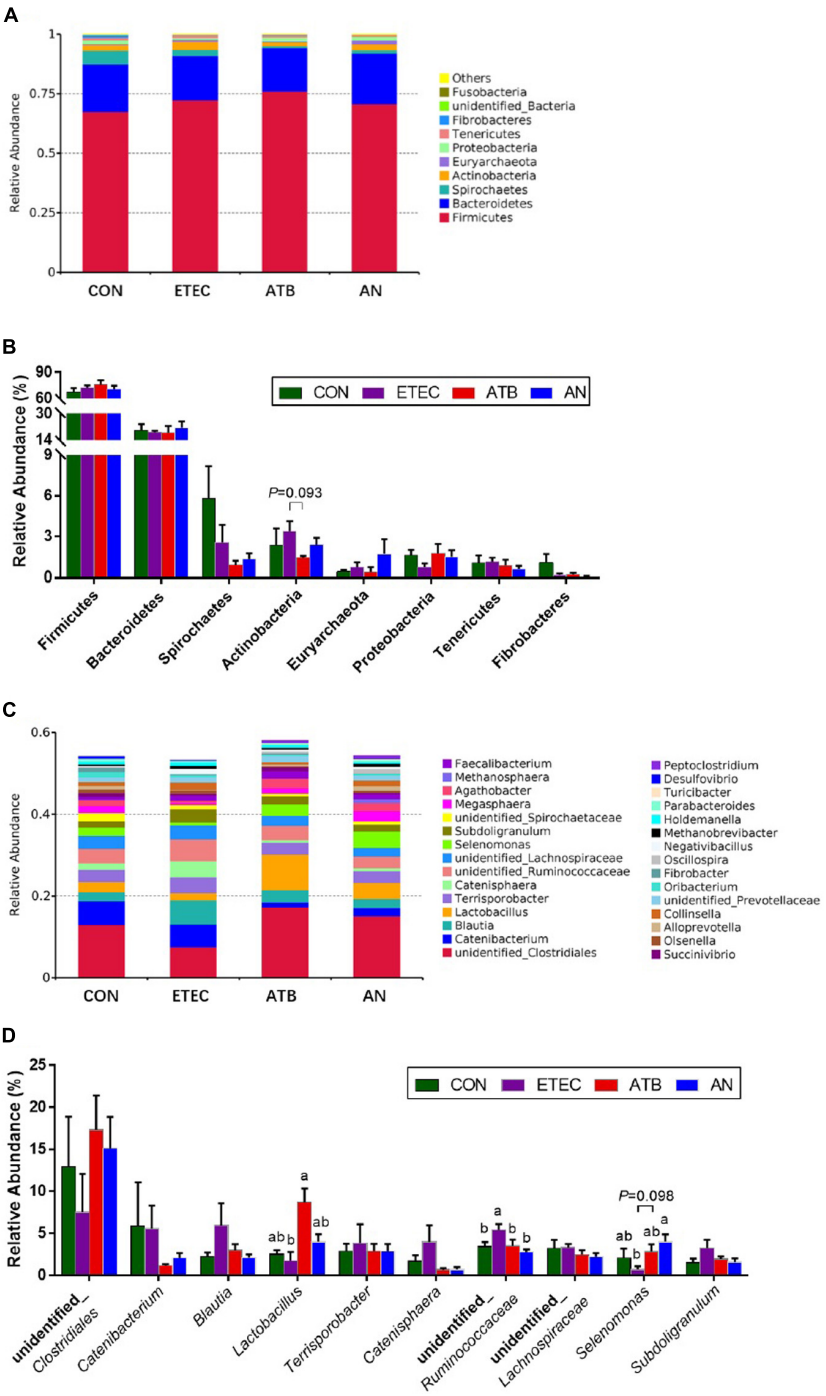

FIGURE 5 | Effect of anethole on microbial composition of fecal microbiota of piglets challenged with enterotoxigenic *Escherichia coli* K88 (n = 5). **(A)** Relative abundance in phyla. **(B)** Top eight in phyla. **(C)** Relative abundance in genus. **(D)** Top 10 in genus.

example, TLR4 proactively identifies the lipopolysaccharide (LPS) of ETEC, while TLR5 and TLR9 recognize flagellin and CpG-DNA, respectively (Cario, 2005; Lu et al., 2008; Kim et al., 2015). The activation of TLRs leads to the secretion of a large number of proinflammatory cytokines via the myeloid differentiation factor 88 (MyD88)/nuclear factor-kappa B (NF-κB) signaling pathway (Kawai and Akira, 2007). The present study showed that seven days after ETEC challenge, the relative mRNA expression of MyD88 in the jejunum was significantly upregulated in the piglets challenged with ETEC. Similarly, TLR5 in the ileum and TLR4, TLR5, TLR9, TRAF6, and NF-κB in the MLN were significantly upregulated. However,

most of these genes (except TLR4 and TRAF6 in MLN) were not altered in the piglets given dietary supplementation of ATB or AN. To maintain the stability of the immune system, the MyD88/NF-κB signaling pathway is negatively regulated by TOLLIP and SIGIRR (Burns et al., 2000; Wald et al., 2003). In this study, the relative mRNA expression of SIGIRR in the jejunum, ileum, and MLN and the relative mRNA expression of TOLLIP in the ileum were upregulated to varying degrees with the administration of AN and ATB. Additionally, no significant difference in the MLN and TOLLIP mRNA expression was identified between the AN and ATB group. These results indicated that the AN supplements had functions similar to those antibiotics, which can inhibit the MyD88/NF-κB signaling pathway by activating its negative regulators. This has shown direct evidence that AN alleviates the inflammation induced by ETEC challenge in piglets.

Activated NF-κB regulates the expression of proinflammatory cytokines (Kawai and Akira, 2007). In the present study, we found that the mRNA expression of IL-1β and TNF-α in the jejunum, ileum and MLN was increased to varying degrees after ETEC challenge. The concentrations of IL-1β and TNF-α in the plasma also tended to increase. Elevated concentrations of IL-1β and TNF-α generate heat and lead to elevated rectal temperature (Yi et al., 2005; Tesch et al., 2018). This observation might partially explain the rapid increase in the intestinal temperature of piglets in response to intestinal infection in our study. Tight junctions (TJs) are the most important connections between cells, TJs only allow soluble, small molecule substances to pass through them, which hinders the passage of macromolecular substances and microorganisms (Lee, 2015), Excessive production of the proinflammatory cytokines IL-1β and TNF-α also break tight junction and increase cell bypass permeability in the gut (McKay and Baird, 1999; Ma et al., 2004; Al-Sadi et al., 2010). In addition, mucin protects the biological function of epithelial cells and participates in the processes of epithelial cell renewal and cell signaling activation, studies have found that downregulated mucin-1 can increase TNF-α expression (Guang et al., 2010). In our study, we observed the disruption of tight junctions and mucin secretion in response to ETEC infection. It is worth noting that AN is not the only essential oil that can regulate the inflammation of the intestine. In previous studies, thymol and oregano were also found to significantly alleviate the increase in IL-1β and TNF-α in the piglet jejunum mucosa caused by challenge with ETEC (Pu et al., 2018).

As we known, intestinal inflammation and gut microorganisms have relations, to investigate the effects of AN on the proliferation of intestinal microbiome in piglets, the microbes in the feces were analyzed by high-throughput 16S rDNA sequencing. In this study, the alpha diversity of the fecal microbiota in the AN group was significantly higher than that in the CON and ETEC group. Beta diversity showed that the microbiota from the ETEC group obviously tended to separate from that of both the ATB and AN group. Thus, the AN group had more similar microbial structures than the ATB group, which is different from the ETEC group. This evidence indicates that AN supplements have functions similar to those of antibiotics in modifying the structure of the intestinal flora. Specifically, we found the ATB group exhibited a significantly increased abundance of *Lactobacillus*, while the AN group exhibited a significantly increased abundance of *Selenomonas*, and both the ATB and AN group exhibited a significantly reduced abundance of unidentified_Ruminococcaceae. Under the normal condition, *Lactobacillus* can inhibit the TLR4 inflammatory signaling triggered by ETEC, which is conducive to the maturation of the intestinal mucosal immune system and triggers local immunomodulatory activity (Zhang et al., 2011; Finamore et al., 2014). *Selenomonas* can produce SCFAs which inhibit inflammation and enhance barrier function (Bladen et al., 1961; Rajilić-Stojanović et al., 2015). Recently study was found that Ruminococcaceae is a biomarker of microbes in oxidative damage and is highly abundant in many intestinal injury models (Zhou et al., 2018). Moreover, several studies have shown that Ruminococcaceae could be involved in recovery after ETEC infection (Salonen et al., 2010; Rajilić-Stojanović et al., 2015). These signs indicated that AN has a positive regulatory effect on intestinal microbiota of piglets infected with ETEC, but its mechanism may be different from the antibiotics. Overall, dietary supplementation with AN enriches the abundance of beneficial flora in the intestines of piglets, which enhances the intestinal functions of piglets and reduces the occurrence of inflammation.

CONCLUSION

In summary, AN can attenuate enterotoxigenic *E. coli*-induced intestinal barrier disruption and intestinal inflammation via modification of TLR signaling and intestinal microbiota, then improving the growth performance of weaned piglets infected by ETEC. Meanwhile, AN is a promising alternative to antibiotics in animal husbandry.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA), https://www.ncbi.nlm.nih.gov/sra, SRR13728343.

ETHICS STATEMENT

The animal study was reviewed and approved by South China Agricultural University Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

QY was the principal investigator that designed the study, wrote the manuscript, carried out the animal trials, sample analysis, data collection, and statistical analysis. JL, YZ, and HQ carried out the animal trials and sample analysis. FC supervised the study. SZ revised the manuscript. WG designed and supervised the study

and revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.647242/full#supplementary-material

Supplementary Figure 1 | The experimental procedure.

Supplementary Table 1 | Composition and nutrient levels of basal diets (as fed-basis).

Supplementary Table 2 | Primer sequences of target and reference genes.

Supplementary Table 3 | The relative abundances of 8 phyla.

Supplementary Table 4 | The relative abundances of 30 genera.

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REVIEW
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Modulatory Effects of Probiotics During Pathogenic Infections With Emphasis on Immune Regulation

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In order to inhibit pathogenic complications and to enhance animal and poultry growth, antibiotics have been extensively used for many years. Antibiotics applications not only affect target pathogens but also intestinal beneficially microbes, inducing long-lasting changes in intestinal microbiota associated with diseases. The application of antibiotics also has many other side effects like, intestinal barrier dysfunction, antibiotics residues in foodstuffs, nephropathy, allergy, bone marrow toxicity, mutagenicity, reproductive disorders, hepatotoxicity carcinogenicity, and antibiotic-resistant bacteria, which greatly compromise the efficacy of antibiotics. Thus, the development of new antibiotics is necessary, while the search for antibiotic alternatives continues. Probiotics are considered the ideal antibiotic substitute; in recent years, probiotic research concerning their application during pathogenic infections in humans, aquaculture, poultry, and livestock industry, with emphasis on modulating the immune system of the host, has been attracting considerable interest. Hence, the adverse effects of antibiotics and remedial effects of probiotics during infectious diseases have become central points of focus among researchers. Probiotics are live microorganisms, and when given in adequate quantities, confer good health effects to the host through different mechanisms. Among them, the regulation of host immune response during pathogenic infections is one of the most important mechanisms. A number of studies have investigated different aspects of probiotics. In this review, we mainly summarize recent discoveries and discuss two important aspects: (1) the application of probiotics during pathogenic infections; and (2) their modulatory effects on the immune response of the host during infectious and non-infectious diseases.

Keywords: antibiotic resistant bacteria, antibiotics alternative, probiotics, pathogenic infections, immunomodulating

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INTRODUCTION

The term probiotic is derived from the Greek word (προβιοτικό: πρό and βἰός) meaning "for life" (1, 2). Probiotics have a very old history since their first description; the first clinical trial investigating the remedial effects of probiotics in constipation was started in 1930 (3). Probiotics have a wide range of applications in poultry, livestock, aquaculture, and also in

humans for the prevention and treatment of disorders, ailments, and infectious and non-infectious diseases (e.g., bacterial, viral, parasitic, or fungal diseases, nervous system disorders, obesity, cancer, and allergic problems), as well as preoperative and postoperative processes. Nowadays, probiotics are an inevitable part of human nutrition with elevated consumption levels through naturally and microbially fermented products with enormous amounts of viable beneficial microbes, such as fermented animal products, fermented fruits and their juices, and various other food products (4). Different probiotics like *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Enterococcus*, *Vagococcus*, *Bacillus*, *Clostridium butyricum*, *Micrococcus*, *Rhodococcus*, *Brochothrix*, *Kocuria*, *Pseudomonas*, *Aeromonas*, *Shewanella*, *Enterobacter*, *Roseobacter*, *Vibrio*, *Zooshikella*, *Flavobacterium,* and some yeasts are commonly used probiotics to control infectious diseases as well as to improve health and quality of aquaculture production (5, 6). The application of specific probiotics culture in the poultry and livestock industry has become very common in recent days. Many economically important poultry diseases like Salmonellosis, Clostridial diseases, Coccidiosis etc., respond positively during probiotics treatment (7). Genus *Bacillus*, *Pediococcus*, *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Aspergillus*, and *Saccharomyces* are usually used in poultry (1).

To increase meat production and inhibit pathogenic growth, antibiotics are usually supplemented in the feed of poultry and livestock leading to the emergence of antibiotic-resistant bacteria. Antibiotic-resistant bacteria are becoming very common, presenting difficulties to the treatment of clinical infections with current chemotherapeutics, thus effective and novel strategies which will enable the host immune system to combat the infections are urgently needed (8). Probiotics exert beneficial effects to their hosts by diverse mechanisms, e.g., antimicrobial peptide (AMP) production, fatty acids production, stabilization of disturbed intestinal microflora, competitive pathogen exclusion, and modulation of host innate and adaptive immune responses (9). Nowadays, strategies using probiotics as an immunomodulator to control infectious diseases have become popular. Antimicrobial effects of probiotics by modulating the innate and adaptive immune responses of hosts have been extensively reported in numerous *in vitro* and *in vivo* studies.

Immune cells or epithelial cells can express a series of pattern recognition receptors (PRRs). The typical PRRs consist of Toll-like receptors (TLRs), retinoic acid-inducible gene-I-like receptors (RLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), and C-type lectin receptors (10). Pathogen-associated molecular patterns (PAMPs) of probiotics interact with PRRs to initiate appropriate signaling pathways for the expression of different genes and subsequent production of immune mediators, which help the hosts to counteract the pathogenic infections (11). Besides these immune remedial effects, probiotics also provide other health-promoting effects on hosts. Indigenous microbiota possess different biological activities extending from anabolism to catabolism of large molecules, resulting in beneficial effects on host health as well as microbiota themselves. Intestinal microflora can ferment endogenous mucus and indigestible diet residues and produce vitamins, such as vitamin K and B (12). The following sections of this review provide a brief introduction to probiotics and discuss the mechanism of probiotic functions and their application during pathogenic infections.

HISTORY OF PROBIOTICS

In the early 1900s, Louis Pasteur asserted that microorganisms were responsible for food fermentation, while Élie Metchnikoff stated that the increased longevity of individuals living in the rural areas of Bulgaria was closely associated with the daily consumption of fermented dairy products, such as yogurt. He claimed that lactobacilli could mitigate the putrefactive effects of gastrointestinal metabolism, which contributed to diseases and aging. Approximately 2,000 years earlier, Hippocrates claimed that "death sits in the bowl" (13). Fermented foods have a long history; fermented milk can be traced back to the Neolithic age. The fermentation of milk was first reported around 10,000 BC in the Middle East and India, and around 7,000–5,000 BC in Egypt, Rome, Greece, and the rest of Europe. The first appearance of soy sauce is estimated around 4,000 BC and 3,000 BC in China, Japan, and Korea; fermented rice first appeared around 2,000 BC in Asia. Fish sauce originated from northern Africa and South East Asia around 1,000 BC. The use of wine possibly started in North Africa around 3,000 BC, and subsequently expanded in the Middle East, Greece, Egypt, and Rome. The use of beer may have started around 7,000 BC in China and probably around 5,000 BC in Mesopotamia (2, 14) (**Table 1**).

SELECTION CRITERIA AND HEALTH BENEFITS OF COMMONLY USED PROBIOTICS

A number of microbes have been used as probiotics. The number of microbial organisms with probiotic characteristics is remarkable. Among them, lactic acid bacteria (LAB) are a group of non-spore forming, Gram-positive rods or cocci with tolerability to markedly low pH; they are fermenters of carbohydrates and use carbon as final electron acceptors. LAB have a wide range of applications and are the most commonly used probiotics (15, 16). They are classified on the basis of their cellular morphology and glucose fermentation mode, into Phylum-Firmicutes, Class-Bacilli, and Order-Lactobacillales. Currently, the LAB genera include *Lactobacillus*, *Streptococcus*, *Leuconostoc*, *Carnobacterium*, *Lactococcus*, *Aerococcus*, *Enterococcus*, *Pediococcus*, *Oenococcus*, *Weissella*, *Alloiococcus*, *Tetragenococcus*, *Dolosigranulum*, and *Vagococcus* (17, 18). The most frequently utilized genera of bacteria used in probiotic formulations are *Lactobacillus*, *Enterococcus*, *Streptococcus, Bacillus*, and *Bifidobacterium*, as well as some fungal strains of the genus *Saccharomyces*, such as *Saccharomyces boulardii* (*S. boulardii*). Most of these are regarded as the intestinal commensal microbiota (2).

The process for the identification of newly-isolated probiotic candidates is the first problem that needs to be addressed. From isolation to market launching, knowledge needs to be collected

TABLE 1 | Some fermented foods history and origin.

Food origin	Aproximate appearance year	Region
Fermented milk	10,000 BC	Middle East
Product of fermented milk	7,000–5,000 BC	Egypt, Italy, Greece
Mushroom	4,000 BC	China
Wine	3,000 BC	North Africa, Middle East, Europe
Soy sauce	3,000 BC	China, Korea, Japan
Fermented honey	2,000 BC	Middle East, North Africa
Fermented rice	2,000 BC	China, Asia
Fermented malted cereals: beer	2,000 BC	China, Middle East, North Africa
Chees	2,000 BC	China, Middle East
Fermented meats	1,500 BC	Middle East
Bread	1,500 BC	Egypt, Europe
Pickled vegetables	1,000 BC	China, Europe
Fish sauce	1,000 BC	Southesat Asia, North Africa
Sourdough bread	1000 BC	Europe
Tea	200 BC	China

on host health, adhesion properties, and resistance to host biochemical environments. Probiotics must be safe, adhere to the lining of intestinal cells with high survival potential, have an immunostimulatory function, have the ability to colonize the tract lumen, withstand exposure to low pH and bile salt, and should have antipathogenic characteristics (19, 20). Other probiotic properties may be considered for selecting probiotic strains with cognitive effects, such as their ability to lower cholesterol, antioxidant function, or cytotoxic impact on cancer cells. Of note, a prospective probiotic does not need to follow or meet all aforementioned selection criteria (21). **Figure 1** shows some properties of good probiotics.

The microbiota inhabiting the animal body provide crucial services to the ecosystem, such as the production of important resources and bioconversion of different nutrients, which are beneficial for both the host and microbes. Microbiota can execute different crucial biological activities, ranging from anabolism to catabolism of large molecules. These biological activities can be beneficial for host health and the microbes. The metabolic functions of intestinal microflora reduce the energy costs of their host, as they ferment endogenous mucus and indigestible food residues, and also produce vitamins such as vitamin K and B (12). Therefore, due to their biological activities, probiotics have positive health effects on hosts, including reduction of the energy required during digestion and provision of beneficial nutrients. Different kinds of commercially available probiotics products are available to boost the health of adults and children (www.probioticchart.ca, www.usprobioticguide.come) (22).

PROBIOTICS ENCAPSULATION

Because of the substantial decrease in their viability in the harsh gastrointestinal environment of the host (gastric pH, protease, lipase, and peristalsis) and during different food processing and storage conditions (high temperature, pH changes, oxygen, and hydrogen), the possible beneficial health effects of probiotics may not be recognized. A number of systems have been designed to improve orally administered probiotics viable number in gastrointestinal tract (GIT), including coating and embedding systems (23). Microencapsulation is an efficient technique that is used to increase the viability and resistance of probiotics against the harsh environmental conditions of GIT and during storage conditions. Microencapsulation is a physicochemical or mechanical process in which probiotics are usually inserted or coated with food-grade materials like lipids, biopolymers, or other hydrocolloidal materials, providing protection against adverse conditions such as heat shock, low pH, bile salts, cold shock, etc. (24). Several studies have been reported that microencapsulation increases the viability of probiotics Encapsulation of *Bifidobacterium longum* with milk increases its viability during storage time (25). *Lactococcus lactis* subsp. *cremoris* LM0230 encapsulation in alginate increases its stability and viability (26). Similarly, *Lactobacillus rhamnosus* GG encapsulation with pectin increases its viability in simulated GIT conditions. Muhammad et al. (27) reported *Lactobacillus plantarum* KLDS 1.0344 ability to alleviate chronic lead toxicity in mice increases when encapsulated with starch originated from tomatoes (27). The study of Riaz et al. (28) shows that the survival rate under simulated GIT conditions of zein-coated alginate *Bifidobacterium bifidum* significantly increases.

POTENTIAL MECHANISMS OF THE PROBIOTIC FUNCTION

The mechanisms of probiotic function are complex, heterogeneous, and specific to probiotic strains. They include competitive exclusion of pathogens (29), ability to colonize the intestine (30), intestinal barrier function improvement by increasing the expression of tight junction proteins and mucin expression along with the interaction of PAMP to PRRs, AMP production (31), and immune system regulation. Some important mechanisms are briefly discussed below.

Competitive Pathogen Exclusion

This refers to a condition in which one bacterial species has a greater potential to attach the epithelia, through a receptor, than other species (11). The known mechanisms of competitive exclusion mainly include lowering the pH in the lumen, contesting for nutrient utilization, and AMP production against competitors (32). Interaction between molecules distributed in the gut epithelia and the surface of bacterial cells mediates the adhesion and colonization of bacteria. Commensal or probiotic bacteria produce adhesive surface molecules (e.g., enolases, glyceraldehyde-3-phosphate, and pyruvate dehydrogenase) and adhere to the extracellular matrix of the host (33, 34). These adhesive surface molecules assist commensal bacteria and probiotics in contesting and preventing pathogenic bacterial attachment and colonization (35, 36). *Lactobacillus fermentum* (*L. fermentum*) competitively binds to collagen I of host epithelial

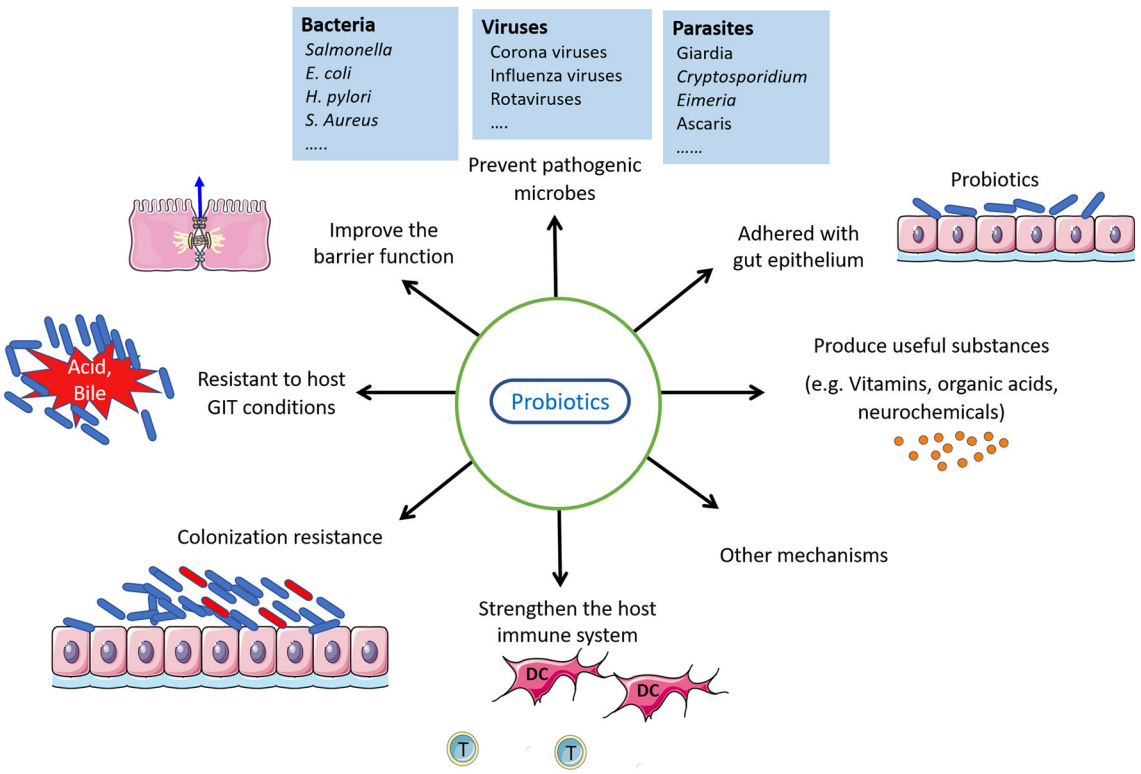

FIGURE 1 | Probiotics properties and their action.

cells by expressing its collagen-binding protein genes and inhibits the binding of *Campylobacter jejuni*. Similarly, *Lactobacillus gasseri* expresses aggregation-promoting factors on their cell surface, which helps in self-aggregation and its binding with the host extracellular matrix fibronectin component. This facilitates the colonization of probiotics and the exclusion of pathogens from the GIT (37). *L. gasseri* also inhibits the adhesion of *Helicobacter pylori* (*H. pylori*) to AGS gastric epithelial cell lines by expressing its Sortase A (srtA) gene, which produces surface molecules that facilitate *L. gasseri* aggregation, as well as binding and adhesion to AGS cell lines (38). Pretreatment with some probiotics impedes pathogenic bacterial attachment to host cell receptor sites by steric hindrance pose, and reduces the colonization of unwanted microbes by producing negative growth factors for pathogens (39). Seaweed *Bacillus* probiotics have good adhesion properties to shrimp intestinal mucosa with competitive exclusion ability and eliminate *Vibrio parahaemolyticus* strain 3HP (40).

Competitive exclusion of probiotics exerts the beneficial effects on the GIT and other organs of the host, increases the adhesion of probiotics, and performs protective actions against pathogens by competing for binding sites of the host. Furthermore, this adhesion of probiotics increases the opportunity for interaction with the host, which favors the immunostimulatory effects of probiotic surface molecules (ligands for receptors of the host) and their metabolites (41, 42). Therefore, the competitive exclusion properties of probiotics offer several benefits to host health, including the reduction of pathogenic attachment, colonization (many diseases arise because of pathogen colonization), further spread of the pathogen, and pathogenic load in hosts. Furthermore, this property of probiotics enables them to colonize the host GIT, which is necessary for the further beneficial action of probiotics to their hosts.

Intestinal Colonization

The potential of probiotics to colonize the intestine is one of the most important properties recommended by WHO/The Food and Agriculture Organization of the United Nations (FAO). The positive characteristics of probiotics, such as antagonisms to harmful microbes or the modulation of the immune system, are linked to their intestinal colonization, which is investigated *in vitro* using simulated intestinal cells, as *in vivo* investigation is difficult (43). The adhesion of LAB with intestinal cells has been extensively reported. Interaction between molecules distributed on gut epithelia and the surface of bacterial cells mediates the adhesion and colonization of bacteria and is highly variable between different bacterial strains. García-Ruiz et al. (44) reported 0.37–12.2% adhesion of wine-isolated LAB (44) and Pisano et al. (45) reported 3–20% adhesion of LAB (45).

Intestinal Barrier Function

As the intestinal epithelial barrier acts as a physical and biochemical barrier and is important for preventing systemic

entry of toxins, bacteria, and other foreign unwanted compounds, so its integrity and full function are quite important. It has been reported in many studies that LAB can improve intestinal epithelial barrier damage induced by pathogenic infection (46–51). Probiotics possess a diverse mechanism of action to improve the intestinal barrier function and maintain homeostasis. "*Lactobacillus* contains a HSP27-inducible polyphosphate (poly P) fraction. Probiotic-derived polyphosphates, strengthen the epithelial barrier function and keep intestinal homeostasis through the integrin-p38 MAPK pathway" (52). *Lactobacillus casei* DN-114 001 and *Lactobacillus acidophilus* strain LB have the potential to improve intestinal epithelial barrier during *Escherichia coli* infection (53, 54). Strains of *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* stimulate tight junction proteins (occludin, claudin-1) results in enhanced barrier stability (55). *L. plantarum* WCFS1 significantly increases occludin and ZO-1 in tight junction vicinity by TLR2 dependent pathway and protect tight junction disruption by toxins, pathogens, and cytokines (49). Qin et al., also showed that *L. plantarum* has protective effects on intestinal barrier by rearranging tight junction proteins (occludin, claudin-1, JAM-1 ZO-1) disturbed by *E. coli* and ameliorates barrier function (50). Another strain of *L. plantarum*, MB452 increases occludin expression and improves intestinal barrier integrity (46). *E. coli* Nissle 1917 (EcN) ameliorates *E. coli* induced intestinal epithelial barrier dysfunction by regulating the expression of occludin and claudin (56). *L. rhamnosus* (LR: MTCC-5897) and *L. fermentum* (LF: MTCC-5898) significantly improve the *E. coli* disturbed tight junction proteins (Occludin, ZO-1, cingulin-1, claudin-1) in Caco-2 cells (57).

Several other reports of Lactobacilli study have also been shown that *Lactobacilli* ameliorate the intestinal barrier damage and pro-inflammatory cytokines production induced by *Salmonella* (47, 58). Probiotics are also effective to improve malnutritional induced intestinal barrier damaged as indicated by the study of Garg et al. on a malnutritional mice model, in which they reported that *Lactobacillus reuteri* LR6 feeding significantly improves the intestinal morphology damaged during malnutrition (59).

Antimicrobial Peptide Production

Different criteria are applied to AMP classification according to their source (animals, fungi, plants, and bacteria), mechanisms of action (AMP acting on cell surface molecules or intracellular components), structure (patterns of covalent bonding), and biosynthetic pathway (non-ribosomally synthesized or ribosomally synthesized) (60). Bacteriocins (AMP from prokaryotes) of LAB are classified into three classes: Class I, post-translationally modified (e.g., lantibiotics); Class II, non-modified, heat stable with size <10 kDa (e.g., pediocin PA1, leucocin A, plantaricin A, and enterocin X); and Class III, heat labile, large peptides with size >30 kDa (e.g., helveticin J) (16). Bacteriocins have low molecular weight and form pores in target cell membranes, leading to the death (61) of pathogenic bacteria, and also act as anti-cancerous agents. Furthermore, bacteriocins also possess immunomodulatory properties with pronounced anti-inflammatory effects during pathogenic infections. As bacteriocins are non-toxic, particularly those derived from LAB, they are used in food preservation. A number of studies showed that certain kinds of probiotics inhibit many types of pathogenic bacteria (*proteus* spp., *E. coli*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Listeria monocytogenes, Citrobacter freundii*, *H. pylori*, *Enterobacter aerogenes*, *Compylobacter jejuni*, *Micrococcus luteus*, *Salmonella* spp., *Shigella* spp., and some fungi) by the action of their bacteriocins (62). Bacteriocins from *Lactobacillus salivarius* inhibit foodborne and other medically important bacteria, such as *Listeria monocytogenes*, many genera of *staphylococcus*, *Neisseria gonorrhoeae*, *Bacillus*, and *Enterococcus*; the bacteriocins kill these bacteria by creating membrane pores and subsequent leakage of cellular material. Further, these bacteriocins also assist *L. salivarius* colonization in the intestine without showing any prominent adverse effects on other lactic acid bacteria (63) *L. plantarum* also exerts antimicrobial activities by producing many types of bacteriocins with antimicrobial effects against food spoilage bacteria, such as *Alicyclobacillus acidoterrestris* (64), *Salmonella* spp., *Listeria monocytogenes*, *Staphylococcus aureus*, and *E. coli*; thus, they may be used as preservatives for pork meat (65). Apart from bacteria, some bacteriocins from *L. plantarum* are also effective against yeast and molds, such as *Fusarium*, *Candida*, *Aspergillus*, and *Mucor* (66). Bacteriocins from other probiotic species markedly induce apoptosis and inhibit tumor formation, cancer cell proliferation, and membrane depolarization of cancer cells during treatment (61). There are different classification systems for AMP and, owing to their diverse mechanism of actions, they have a wide range of applications in humans and animals, as well as aquaculture fields (67). They inhibit growth and even kill diverse pathogens by creating pores in their cell membranes, as well as initiating appropriate immune responses.

Immune Regulation

It is well-established that probiotic bacteria exert an immunomodulatory effect and have the potential to communicate and interact with a series of immune cells (e.g., DCs, lymphocytes, epithelial cells, monocytes, and macrophages). The immune response generally comprises the innate immune response and adaptive immune response. Innate immune response responds to PAMPs distributed on the majority of bacteria (11). The principle immune response to any pathogen is activated following the interaction of PRRs (i.e., TLRs, NLRs, and C-type lectin receptors) with PAMPs and initiates cell signaling. Intestinal epithelial cells are the host cells that most extensively come into contact with probiotics. However, probiotics may also interact with DCs, which play a significant role in the innate immune response and bridge the innate and adaptive immune responses. Through their PRRs, both intestinal epithelial cells and DCs can communicate and react to gut microorganisms (68, 69). Under the effects of probiotics/commensal microbiota, the activated DCs induce the appropriate immune response (e.g., naïve CD4 T cells to Treg cell differentiation), which generally inhibits Th1-, Th2-, and Th17-mediated inflammatory response. Furthermore, probiotics blunt intestinal inflammation (70) by downregulating the expression

of TLRs *via* secretion of TNF-α inhibitory metabolites and inhibition of nuclear factor-κB (NF-κB) signaling in enterocytes (68). Probiotics also modulate the expression of various kinds of cytokine production.

Cytokines Mediated Immune Response and Probiotics

Probiotic benefits related to immunoregulation for the treatment of various diseases have been extensively studied. Immunomodulatory effects of probiotics are mainly due to the induction of the release of cytokines including interleukins, transforming growth factor (TGF), tumor necrosis factors (TNFs), interferons (INFs), and immune cells released chemokines, which further regulate the immune system (71, 72). Immunostimulatory and immunoregulatory actions of probiotics have been reported in various studies. Immunostimulatory probiotics are capable of acting against infection and cancer cells, inducing the release of IL-12, which stimulates the NK cells and produces the Th1 cells. By maintaining the balance between Th1 and Th2, these probiotics also work against allergies. Contrary to this finding, immunoregulatory probiotics are attributed to Treg cells and IL-10 production to blunt excessive inflammatory responses, inflammatory bowel disease, and autoimmune disorders (73, 74). So, probiotics immunomodulatory effects *via* cytokines are strain-specific as indicated by the *in vitro* study of Haller et al. (75) using Caco-2 cells in which they reported that *Lactobacillus sakei* is capable of inducing pro-inflammatory cytokines (IL-1β, TNFα, and IL-8) whereas *Lactobacillus johnsonii* induced anti-inflammatory cytokines (TGF-β) (75). A mixture of *Lactobacillus paracasei* and *L. reuteri* to *Helicobacter hepaticus* IL-10-defcient mice leads to reduced colitis and pro-inflammatory cytokines production (76). Kourelis et al. (77) study on Fisher-344 inbred rats and BALB/c, demonstrated that *L. acidophilus* NCFB 1748 and *L. paracasei* subsp. *Paracasei* DC412 induce specific immune markers and innate immune responses *via* recruiting polymorphonuclear cell and production of TNFα (77). Probiotics-induced cytokines expression for immune system modulation of the host has been briefly discussed in the relevant section.

Toll-Like Receptor-Mediated Immune Response and Probiotics

Toll-like receptors and single-pass membrane-spanning receptors are very important protein receptors expressed on several non-immune (epithelial, fibroblasts) and immune [macrophages, B cells, natural killer (NK) cells, DCs] cells. Activation of the TLR signaling pathway, except TLR3 (78), generally leads to the recruitment of MyD88, which results in activation of the NF-κB and mitogen-activated protein kinase (MAPK) pathway. TLR-induced signaling is also responsible for the maturation of DCs characterized by increased expression levels of DC markers (CD80, CD83, and CD86) and chemokines receptor C-C motif chemokine receptor 7 (CCR7). TLR9 is crucial for the mediation of the anti-inflammatory effects of probiotics, though many other receptors are also involved.

Lactobacilli ligands initiate cell signaling after binding to TLR2 in combination with TLR6, endorsing dimerization and NF-κB activation *via* recruitment of MyD88 (79). Engagement of a bacterial ligand with TLR2 results in cytokine production and increases the transepthelial resistance for conquering microbes (79, 80). Several *Lactobacillus* strains induce their immunomodulatory effects by binding to TLR2, which recognizes peptidogycan (a component of the cell wall of Gram-positive bacteria). An *in vitro* study showed that *L. plantarum* and *L. rhamnosus* increased TLR2 expression in human cells (Caco-2). *L. casei* showed similar effects in *Salmonella*-infected and healthy mice, and induced TLR expression, as well as interleukin-10 (IL-10), interferon-gamma (IFN-γ), and TNF-α production (81, 82).

Numerous other probiotics interact with TLR4 to induce an appropriate immune response. For example, during pre- and post-*Salmonella* challenges in mice, *L. casei* increased the production of IL10, IFN-γ, and IL6, and reduced the levels of TNF-α by interacting with TLR4 (82, 83). Likewise, *L. rhamnosus* GG (heat-inactivated) and *Lactobacillus delbrueckii* subsp. *Bulgaricus* (*L. delbrueckii*) reduce TLR4 expression in DCs (human monocyte-derived) (84). TLR9, another important TLR, identifies bacterial CpG DNA and CpG-ODN (engineered unmethylated oligonucleotide mimics). Unmethylated pieces of DNA comprising CpG patterns produced from probiotics also have the propensity to mediate anti-inflammatory activities *via* TLR9.

In the case of the differentiated epithelium, apical, and basolateral stimulation results in the activation of different signaling pathways. Basolateral TLR9 activation causes activation of the NF-κB cascade by the degradation of IκBα. Of note, apical activation of TLR9 results in the suppression of NF-κB by the aggregation of ubiquitinated IκB in the cytoplasm (85). Apical or basolateral stimulation of these receptors is important and involves different signaling cascades leading to various immune responses. The results from the study conducted by Ghadimi et al. show that polarized T84 and HT-29 cells increase TLR9 expression in a specific manner in response to apically applied natural commensal origin DNA. They reported that when LGG DNA is applied to these cells, it attenuates TNF-α enhanced NF-κB activity by reducing IκBα degradation and p38 phosphorylation (86).

Lactobacillus plantarum-purified DNA also modulates the immune response of host cells by interacting with TLRs, as reported by Kim, whose studies show that *L. plantarum*-purified DNA inhibits LPS induced TNF-α production in THP-1 cells. Furthermore, *L. plantarum*-purified DNA blunt the expression of TLR4, TLR2, and TLR9, which induce NF-κB activation through the LPS signaling pathway, leading to pro-inflammatory cytokines upregulation (87, 88). TLRs are important membrane receptors; most intracellular signaling pathways involve the activation of membrane receptors. Furthermore, TLRs play a key role in the induction of immune response by probiotics through the recruitment of specific intracellular signaling molecules. Depending on their interaction with specific TLRs, probiotics may decrease or increase TLR expression.

NLR-Mediated Immune Response and Probiotics

In tissues with blunt TLR expression, NLRs are important and present in the cytoplasm. Thus far, more than 20 NLRs have been recognized. Among them, NOD1 and NOD2 are the most studied and important NLRs (89). NOD1 is expressed in many cells and recognizes peptidoglycan moieties (comprising Gram-negative meso diaminopimelic acid). NOD2 is mainly expressed on DCs, lungs cells, macrophages, intestinal cells, buccal epithelium, and Paneth cells. It senses muramyl dipeptide motifs which are present in a wide range of bacteria (90). NOD1 and NOD2 undergo self-oligomerization following recognition by their agonist. This results in the recruitment and activation of receptor interacting serine/threonine kinase 2 (RICK; an adaptor protein, kinase responsible for the regulation of apoptosis *via* CD95), which is necessary for MAPKs and NF-κB activation and the subsequent production of inflammatory mediators such as cytokines and chemoattractants. Another important signaling factor that NLRs trigger is, apoptosis-associated speck-like protein with caspase induction to trigger caspase 1 (CASP1; an adaptor protein required for the functionally effective and mature forms of pro IL18 and pro IL1). NLRs are involved in the formation of the inflammasome that results in CASP1 activation. There are three major inflammasomes named according to the NLRs involved: NOD-like receptor family pyrin domain containing protein 1 (NLRP1), NLRP3, and NLRC4. Murymyl dipeptide, bacterial and viral RNA, and lipopolysaccharides are sensed by NLRP3 (91). Many *Lactobacillus* species exert their immune regulatory effects *via* NLRs. In galactose-1-phosphate uridylyltransferase (GALT) of swine, *L. gasseri* and *L. delbrueckii* increase the expression of NLRP3 *via* TLR and the NOD signaling cascade, leading to appropriate activation of NLRP3. Furthermore, NOD1, NOD2, TLR2, and TLR9 agonists also enhance NLRP3 expression. *L. salivarius* exerts its anti-inflammatory effect by producing IL10 *via* regulation of NOD2 (92, 93). Probiotics modulate systemic and local immune responses of the host in a strain-specific manner by the expression of PAMPS, such as flagellin, lipopolysaccharides, CpG-DNA, and other surface proteins. PAMPs are recognized by PRRs expressed on numerous immune and epithelial cells. TLRs, C-lectin type receptors, and NLRs are the best studied PRRs. PRRs have broad specificity and their limited number can recognize a wide range of PAMPs. Interaction between PAMPs and PRRs results in the activation of multiple molecular signaling cascades that generate a specific cellular response against the encountered microbes.

Probiotics and Regulation of the NF-κB Pathway

The NF-κB pathway is involved in many pathological conditions and controls the expression of many (∼150) pro-inflammatory and anti-inflammatory cytokines genes. These genes are extensively involved in both adaptive and innate immune responses. NF-κB is found in nearly all types of cells (94, 95). Many probiotics regulate the activation of the NF-κB pathway. *L. casei* inhibits *Shigella fexneri*-induced activation of the NF-κB pathway (96). *L. rhamnosus* and *Lactobacillus helveticus* downregulate the Th1 pro-inflammatory response and improve Th2 response during *Citrobacter rodentium* infection (97). *Bifdobacterium lactis* inhibits IκBα degradation during colitis (98). Some researchers have claimed that dietary yeast downregulates TLR2, NF-κβ p65, MyD88, IL8, and IL1β (99). *L. reuteri*, *L. casei*, and *L. paracasei* show anti-inflammatory characteristics *via* NF-κB pathway regulation; for example, *L. reuteri* decreases the expression of inflammatory mRNA cytokines production and increases anti-inflammatory cytokines production, and also improves the production of apoptosis-inhibiting proteins to improve cell survival and its immune response. *L. reuteri* do this by interfering the ubiquitination of IκB and nuclear translocation of p65 (NF-κB subunit), respectively (100–102). *L. casei* and *L. paracasei* hinder the production of pro-inflammatory cytokines by inhibiting the phosphorylation of IκBα and nuclear translocation of p65, and also reverse the degradation of IκBα (103, 104). Similar inhibitory effects on the NF- κB pathway have been shown by *L. plantarum* and *L. brevis*. *L. plantarum* inhibits NF-κB-activating factors by decreasing the binding activity of NF-κB (105), while *L. brevis* prevents interleukin 1 receptor associated kinase 1 (IRAK1) and AKT phosphorylation (106). *Bifdobacterium infantis* and *Streptococcus salivarius* also reduce NF-κB activation (101).

Besides these probiotics have several other mechanisms of action related to antifungal, antibacterial, antiviral, antiparasitic, antiallergic, anti-cancerous, antidiabetic, amelioration of the cardiovascular system, the reproductive system, and the central nervous system which has been briefly discussed in the relevant section.

IMMUNE REGULATION-BASED THERAPEUTIC APPLICATION OF PROBIOTICS DURING INFECTIOUS DISEASES

Probiotics have a wide range of applications covering numerous non-infectious and infectious diseases, including bacterial, viral, parasitic, fungal, and many other non-infectious diseases. They exert anti-pathogenic effects by modulating both the innate and adaptive immune responses of the host.

Bacterial Diseases

Due to the several disadvantages associated with the preventive use of antibiotics, strict controls have been introduced to prohibit or reduce their use during the treatment of bacterial diseases. In the last three decades, the dietary application of feed additives has been attracting attention as a replacement for antibiotics. Probiotics have been among the most effective feed additives for the control or treatment of bacterial diseases (5). Immune modulatory therapies with probiotics for some selected pathogens are briefly discussed below (**Table 2**).

Salmonella Infection

Probiotics may be used as alternatives to the prophylactic use of drugs for the control and prevention of salmonellosis (137). *Salmonella* causes a foodborne disease in both animals and humans with high morbidity (93.8 million human infections) and mortality (155,000 deaths) worldwide annually (138–142).

TABLE 2 | Probiotic therapies during bacterial diseases.

Probiotics	Target bacteria	Study models	Mechanism of action	Effects	References
L. rhamnosus S1K3	*S.* Typhimurium	Caco-2 cells, mice	↑ Claudin-1 ↑ sIgA, sIgA secreting cells Maintain IL-4, IL-12 protein level ↓ TGFβ	↑ Barrier integrity ↓ *Salmonella* count Improves health status	(107)
Multistrain formula consisting of different *Lactobacilli*	*S.* Typhimurium	Chicken	↓ IFN-γ production	↓ *Salmonella* complications ↑ Recovery rate	(47)
L. plantarum LPZ01	*S.* Typhimurium	Chicken	↓ IFN-γ production Regulate miRNA	↓ *Salmonella* load and associated complications	(108)
L. casei DBN023	*S. pullorum*	Chicken	↓ TNF-α and IFN-γ ↑ IL10	↑ Villi height ↑ Muscle thickness ↑ Intestinal immune functions ↓ Mortality ↓ Pathological changes ↓ Inflammation	(58)
L. casei CRL 431	*S.* Typhimurium	Mice	↑ IL10	↓ *Salmonella* associated complications	(82)
S. cerevisiae strain 905	*S.* Typhimurium	Mice	↑ IgA, IgM in serum ↑ Kupffer cells in liver ↓ IL-6, TNF-α, and IFN-γ	↓ *Salmonella* load in Peyer's patches, spleen, mesenteric lymph nodes, liver ↓ Mortality	(109–111)
S. boulardii	*S.* Typhimurium	T84 cells	↓ NF-kB, MAPKs ERK1/2, p38, and JNK activation ↓ IL-8	↓ *Salmonella* associated complications	(112)
L. gasseri Kx110A1	*H. pylori*	THP-1 cells	↓ TNF-α, IL6	↓ *Salmonella* associated complications	(113)
L. fermentum UCO-979C	*H. pylori*	AGS cells	↓ IL8, IL1β, MCP-1	↓ *H. pylori* induced gastric inflammation	(114)
L. acidophilus and *L. rhamnosus*	*H. pylori*	AGS cells	↓ NF-κB and MAPK activation ↓ IL8, IL6, MAP-2, IL1β, TNF-α.	↓ *H. pylori* induced gastric inflammation	(115–117)
L. bulgaricus NQ2508	*H. pylori*	GES-1 cells	↓ TLR4 expression ↓ NF-κB activation ↓ IL8	↓ *H. pylori* induced gastric inflammation	(118)
L. rhamnosus GG	*H. pylori*	AGS and Caco-2 cells	↓ Gastrin-17 ↓ IL8 and TNF-α	↓ *H. pylori* induced gastric inflammation and ulceration	(119)
L. paracasei 06TCa19	*H. pylori*	MKN45 cells	↓ NF-κB and p38 MAPK activation ↓ IL-8 and RANTES	↓ *H. pylori* induced gastric inflammation and ulceration	(120)
S. boulardii	Clostridial infection	BALB/c mice	↑ IgA, IgG, IgM	↓ Clostridial infection severity	(121)
S. boulardii	Clostridial infection	Mice	Inhibits the *Clostridium* toxins A-induced ERK1/2 and JNK/SAPK signaling pathways	↓ Clostridial infection severity	(122)
S. boulardii	Clostridial infection	Rat	Degrades Clostridial toxins by its protease action ↓ Binding of toxins to host cell	↓ Clostridial infection severity	(123)
L. casei BL23	*S. aureus*	Bovine mammary epithelial cells	↓ IL8, IL6, TNF-α, IL1β, and IL1α	↓ Inflammation of the mammary glands	(124)
B. subtilis DS991 EPS	*S. aureus*	C57BL/6J mice	↓ Pro-inflammatory cytokines, chemokines and T-cell activation	↓ Inflammation	(125)
L. salivarius BGHO1	*L. monocytogenes*	Rats	↑ CD14, TNF-α, IL1β ↓ *Listeria* toxins	↑ Protection against *Listeria monocytogenes*	(126)
L. delbrueckii UFV-H2b20	*L. monocytogenes*	Mice	↑ TNF-α and IFN-γ Stimulates macrophages to increase bacterial killing	↑ Lifespan ↓ Bacterial load from liver and spleen ↓ Liver immunopathology	(127)
Heat-killed *Enterococcus faecium* BGPAS1-3 cell wall protein	*L. monocytogenes*	Caco-2 cells	↑ TGF-β and claudin production ↑ TLR4 expression ↓ TLR2 expression	↓ *Listeria monocytogenes* infection	(128)

(Continued)

TABLE 2 | Continued

Probiotics	Target bacteria	Study models	Mechanism of action	Effects	References
Enterococcus faecium JWS 833	*L. monocytogenes*	Mice and peritoneal mouse macrophages	↑ TNF-α, IL1β, Nitric oxide (NO)	↓ *Listeria monocytogenes* complications	(129)
L. fermentum MTCC 5898	*E. coli*	Mice	↑ IFN-γ, TFNα, MCP-1 ↑ IgA, IgG1 ↑ Antioxidant enzymes activity ↓ IL-4 and IL-10	↓ *E. coli* load in liver, spleen, intestine, and peritoneal fluids	(167)
L. rhamnosus MTCC 5897	*E. coli*	Mice	↑ IgA, IgG ↑ Antioxidant enzymes activity	↓ *E. coli* load in liver, spleen	(168)
L. rhamnosus (LR: MTCC-5897)	*E. coli*	Caco-2 cells	↑ Claudin-1, Occludin, ZO-1, Cingulin	↓ Hyperpermeability Maintains barrier integrity	(170)
L. fermentum (LF: MTCC-5898)	*E. coli*	Caco-2 cells	↑ Claudin-1, Occludin, ZO-1, Cingulin	↓ Hyperpermeability Maintains barrier integrity	(57)
L. rhamnosus ACTT 7469	*E. coli*	pig	↓ TLR4 ↓ TNF-α, IL8	↓ *E. coli* associated inflammation	(130)
L. plantarum B1	*E. coli*	chickens	↓ TLR4 expression ↓ IL2, IL4, IFN-γ ↑ Mucosal antibodies (IgA)	↓ *E. coli* associated inflammation	(131, 132)
L. jensenii TL2937	*E. coli*	PIE cells	↓ IRAK-M, BCL3, TOLLIP, A20	↓ *E. coli* associated inflammation	(133)
L. amylovorus DSM 1669	*E. coli*	Caco-2 cells and pig explant	Modulates Tollip and IRAK-M ↓ TLR4 expression ↓ phosphorylation of the IKKα, IKKβ, IκBα, and NF-κB subunit p65 ↓ IL-1β and IL8 production ↑ Hsp72 and Hsp90	↓ *E. coli* associated inflammation	(134)
L. delbrueckii TUA 4408	*E. coli*	PIE cells	↓ MAPK and NF-κB activation	↓ *E. coli* associated inflammation	(135)
L. rhamnosus ATCC 7469	*E. coli*	IPEC-J2 cell model	↑ ZO-1 and Occludin ↓ *TLR4* and *NOD2* mRNA expression	Maintain epithelial barrier ↓ *E. coli* associated deleterious effects	(136)

After attachment and internalization into the lamina propria, *Salmonella* induces an inflammatory response, e.g., release of pro-inflammatory cytokines, followed by inflammation, ulceration, diarrhea, and destruction of the mucosa (143). Persistent infection is established due to the ability of *Salmonella* to evade the host immune system (144). The persistence of infection is further aided by virulent factors of *Salmonella* that are responsible for the clonal deletion of CD^+ T cells (145).

When administered in adequate amounts, probiotics have the ability to modulate the expression of immune-related cytokines, including interleukins IL4, IL6, IL12, IFN-γ, and IL1β in lymphoid cells during *Salmonella* infection (47, 107, 108, 142, 146). *L. rhamnosus* S1K3 maintains IL-4 and IL-12 protein levels and reduces TGFβ during the late stage of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) NCDC infection in mice and also increases the level of IgA secreting cells in lamina propria, IgA in serum, and secretory IgA level in intestinal fluids during *S.* Typhimurium NCDC infection in mice. This probiotic also reduces the *S.* Typhimurium NCDC count in feces, prevents its further spread in the liver, spleen, and intestine of mice, and improves overall health. Furthermore, in an *in vitro* study on Caco-2 cells, *L. rhamnosus* S1K3 improves the tight junction proteins (occludin and claudin-1) (107). The production of IFN-γ, a pro-inflammatory cytokine, is induced by *Salmonella*. IFN-γ delays recovery from intestinal inflammation, boosts inflammatory mediators [TNF, ILβ, inducible nitric oxide synthase (iNOS)], and hampers IL22- and lectin REGIIIβ-mediated antimicrobial defense (147). Probiotics beneficially regulate the immune response of the host and suppress the expression of pro-inflammatory cytokines and subsequent inflammation. IFN-γ is suppressed by the anti-inflammatory action of probiotics, greatly reducing the severity of *Salmonella* infection. During salmonellosis, immune players, macrophages, and monocytes secrete IL6, which serves as a pro-inflammatory cytokine and its expression levels are reduced by *Lactobacillus* spp. for the effective and rapid prevention of *Salmonella* infection in broiler chickens (47). A study conducted by Chen et al. showed that *L. plantarum* (LPZ01) reduces *S.* Typhimurium load, IFN-γ expression, TNF-α level, and associated inflammation in broiler chickens by regulating the expression of certain miRNAs involved in immune regulation and inflammatory responses (108). Supplementations with some probiotics increase the activation of B cells and antibody production by increasing IL10 expression. The latter is an important immunoregulatory and anti-inflammatory cytokine involved in antibody production during *Salmonella* infection. *L. casei* (DBN023) improves, regulates, and enhances intestinal immune functions, while cytokines balance and reverse the

detrimental effects of *Salmonella pullorum*, characterized by higher levels of anti-inflammatory cytokines (IL10) and lower levels of pro-inflammatory cytokines (TNF-α, IFN-γ, and IL17). During prophylactic feeding of probiotics in chicken infected by *Salmonella pullorum*, *L. casei* (DBN023) increases villi height and muscle thickness and reduces *Salmonella pullorum*-associated mortality and pathological changes in intestinal epithelial tissues (58). *L. casei* CRL 431 also increases the expression of IL10 to reduce the severity of *S.* Typhimurium infection in BALB/c mice (82). In this manner, probiotics improve the host immune response by hampering the overexpression of inflammatory cytokines, as well as increasing the expression of anti-inflammatory cytokines and production of anti-*Salmonella* antibodies to blunt the severity of *Salmonella* infection.

Some yeasts are also used as immunobiotics and are effective in reducing *Salmonella* infection. The study by Martins et al. shows that *Saccharomyces cerevisiae strain* 905 (S. *cerevisiae* 905) protects and reduces the mortality of mice, orally challenged by *Salmonella* Typhimurium (109), and also significantly reduces the translocation of *S.* Typhimurium to the liver of gnotobiotic mice, and to other organs (Peyer's patches, the spleen, the mesenteric lymph nodes, and the liver) of the conventional mice. The same author in another study shows that this strain increases the number of Kupffer cells in the liver and induces a higher level of secretory IgA in the intestinal contents and IgA and IgM in the serum of mice (110). Furthermore, this strain reduces pro-inflammatory cytokines (IL-6, TNF-α, and IFN-γ) levels and modulates activation of MAPK (p38 and JNK, but not ERK1/2), NF-κB and activator protein-1, signaling pathways which are involved in transcriptional activation of pro-inflammatory mediator during *Salmonella* infection (111). Another yeast strain *S. boulardii* reduces *S.* Typhimurium induced IL-8 production in T84 cells by exerting its inhibitory effects on *S.* Typhimurium induced activation of the MAPKs ERK1/2, p38, and JNK as well as on activation of NF-kB (112). *S. boulardii* possesses the capability to bind with *S.* Typhimurium leading to reduced organ translocation of this pathogen, which results in decreased activation of MAPK (p38, JNK, and ERK1/2), phospho-IkB, p65-RelA, phospho-jun, and c-fos in the colon and signal pathways, involved in the activation of inflammation, induced by *S.* Typhimurium kB (148). Therefore, yeast can survive in host GIT, colonize there, reduce the pathogenic load from the host, and can modulate the immune response of their hosts toward a beneficial pattern.

A series of studies show that short-chain fatty acids (SCFAs) exert diverse beneficial effects on the health of the host gut and body (e.g., anti-inflammatory effects, prevention of histone deacetylases, and suppression of NF-κB resulting in IL1β downregulation), and play a vital role in maintaining intestinal homeostasis. Many probiotics possess regulatory properties for SCFA and can directly or indirectly increase their production. *L. acidophilus* reduces *S.* Typhimurium-induced inflammation directly by increasing the production of SCFA and indirectly by increasing that of other SCFA-producing gut bacteria (149). Moreover, *L. acidophilus* balances *Salmonella*-induced dysbiosis in infected mice (150).

Other probiotics have also shown beneficial effects on the prevention of *Salmonella* infection and inhibit the pathogenesis of *Salmonella* at initial steps. *L. plantarum* (MTCC5690) improves the intestinal defense through modulation of TLR2 and TLR4, and prevents the colonization and further spread of *Salmonella* in mice (151). Similarly, *E. faecium* (PXN33) in combination with *L. salivarius* (59) also inhibits *Salmonella Enteritidis* colonization in the GIT of poultry (152). Supplementation of probiotics greatly reduced the severity of *Salmonella* infection by their immunomodulatory mechanisms of action. As probiotics decrease the expression of inflammatory cytokines and increase the antibody production and anti-inflammatory cytokine expression during salmonellosis, supplementation can improve the overall health of the host.

Helicobacter Pylori Infection

Helicobacter pylori, a Gram-negative and spiral-shaped pathogenic bacterium, resides in >50% of the population worldwide and causes different diseases characterized by prominent gastric inflammation which is associated with gastric ulcers. The mechanism of *H. pylori*-induced inflammation includes chemokine (IL8)-mediated infiltration of neutrophils, increased RANTES level, and *H pylori* urease-induced degradation of NF-κB inhibitor (IκBα) (115, 120, 153–155). *H. pylori* can survive inside macrophages, arrest phagocytosis, and induce their apoptosis by preventing nitric oxide (NO) production. Furthermore, *H. pylori* stimulates macrophages to secret TNF-α and IL6, which are associated with gastric inflammation, by expressing the TNF-α-converting enzyme17 (ADAM17). ADAM17 is a crucial enzyme for the maturation and functioning of TNF-α and IL6. *L. gasseri* Kx110A1 inhibits these pro-inflammatory cytokines from *H. pylori*-infected THP-1 cells by inhibiting the expression of the *H. pylori* ADAM17 enzyme (113). *L. fermentum* UCO-979C regulates the immune response of host macrophages (HumanTHP-1 cell line) and human gastric epithelial cells (AGS cell line) by stimulating them to secrete specific cytokines and chemokines. Moreover, it significantly increases the secretion of inflammatory cytokines (IL6, TNF-α, and IL1β) in both AGS and macrophages, and the secretion of IL10, IFN-γ, and IL12p70 only in macrophages prior to *H. pylori* challenge. In contrast, it decreases the levels of *H. pylori*-induced inflammatory cytokines [IL8, IL1β, monocyte chemoattractant protein-1 (MCP-1), and IL6] in AGS, and those of TNF-α in both AGS and macrophages. Thus, prior to infection, treatment with *L. fermentum* UCO-979C increases inflammatory cytokines to counter future infections. In contrast, during infection, *L. fermentum* UCO-979C treatment lessens the over-activated immune response of host cells, as also shown by Garcia-Castillo et al. (114). The study reported that *L. fermentum* has the ability to decrease *H. pylori*-associated inflammation by improving TGF-β production in the AGS cell line. TGF-β inhibits NF-κB activation by upregulating the levels of IκBα. Notably, *H. pylori* infection impedes this TGF-β-associated signaling pathway by inducing SMAD7 expression to promote inflammation.

Similar to *L. fermentum, L. acidophilus*, and *L. rhamnosus* also regulate the immune response of host cells and decrease their pro-inflammatory immune response against *H. pylori*. As

shown by their anti-inflammatory effects in AGS cells, in which both probiotics greatly reduced the CagA-induced expression of IL8 by inhibiting its translocation into host cells. CagA is an *H. pylori* virulent factor responsible for inflammation by the degradation of cytoplasmic IκBα and increasing translocation of NF-κB into the nucleus (116, 156, 157). Moreover, *L. acidophilus* activates Th1 response to counter *H. pylori* infection, suppresses *H. pylori*-induced SMAD7 expression as well as the activation of the NF-κB and MAPK signaling pathways, and decreases subsequent inflammatory response (production of IL8, IL6, MAP-2, IL1β, TNF-α, and granulocyte-colony stimulating factor) during *H. pylori* infection (115, 117). *L. bulgaricus* NQ2508 also shows similar anti-inflammatory effects by reducing *H. pylori*-induced IκBα degradation and subsequent IL8 production in the human gastric epithelial cell line-1 (GES-1). It may also secrete some soluble proteins which exert inhibitory effects on TLR4 and inhibit its activation by *H. pylori*. Moreover, it blocks subsequent signaling pathways toward NF-κB activation and its delivery to the nucleus for the transcription of pro-inflammatory cytokines (118). As mentioned above, gastric ulcers and cancer are prominent complications of *H. pylori* infection. They mainly arise due to the over-immune response of host cells and the subsequent production of inflammatory cytokines, which are involved in gastric ulceration. Many probiotics reduce these complications by regulating the *H. pylori*-disrupted immune response. *L. rhamnosus* GG reduces gastric ulceration and cancer induced by *H. pylori via* the IL8/TNF-α/Gastrin-17 pathway. *H. pylori* upregulates Gastrin-17 by increasing the levels of IL8 and TNF-α, which in turn upregulate Gastrin-17. Gastrin-17 typically causes gastric cancer, whereas IL8 and TNF-α cause inflammation and apoptosis leading to ulceration of the stomach. *L. rhamnosus* GG shows significant immunobiotic properties with anti-inflammatory effects and attenuates Gastrin-17 levels by suppressing the expression of IL8 and TNF-α (119, 158–161). Similarly, *L. paracasei* may ameliorate *H. pylori*-induced gastric inflammation by regulating the immune response of host cells. *L. paracasei* 06TCa19 inhibits *H. pylori* CagA-induced p38 and IκBα phosphorylation and increases the levels of these NF-κB inhibitors in MKN45 cells. This results in inhibition of the transcription of the inflammatory chemokine genes (120). Numerous other probiotics are extensively used to ameliorate *H. pylori*-induced complications with the aim to regulate the immune system of the host (162, 163).

Escherichia Coli Infection

Escherichia coli causes different problems for humans and animals. Enterotoxigenic *E. coli* (ETEC) causes diarrhea in piglets and other species by secreting heat-labile and heat-stable toxins. Through a complex mechanism, these toxins activate the chloride channel (cystic fibrosis transmembrane channel) resulting in diarrhea. The *E. coli* causing postweaning diarrhea mostly carries F4 (K88) fimbriae (164). $F4^+$ ETEC increases the expression of membrane and cytoplasmic-associated receptors (TLRs and NLRs), which are involved in the NF-κB signaling pathway and subsequent production of pro-inflammatory cytokines (IL8 and TNF-α) leading to inflammation (130, 164, 165).

Probiotics greatly reduce the expression of these pro-inflammatory cytokines by reducing the interaction of *E. coli* with membrane receptors. *L. rhamnosus* ACTT 7469 weakens the *E. coli*-induced expression of TLR4, TNF-α, and IL8 at the protein and mRNA levels in piglets. Furthermore, *L. rhamnosus* increases the expression of TLR2, TLR9, and NLR in the case of *E. coli* infection in piglets, which results in decreased intestinal inflammation (130). As mentioned above, TLR2 and TLR9 are involved in the anti-inflammatory effects of many probiotics.

Similar anti-inflammatory effects have also been shown by supplementation of *L. plantarum* B1, which reduces *E. coli*-induced inflammation in broiler chickens by decreasing the expression of TLR4 and the levels of cytokines (IL2, IL4, and IFN-γ) involved in inflammation. *L. plantarum* also increases the levels of mucosal antibodies (IgA) (131, 132). Hence, probiotics (mainly, the *Lactobacillus* species), regulate the immune response in a beneficial manner by decreasing the expression of membrane receptors (TLR4) involved in inflammation associated with pathogens. On the other hand, probiotics increase the expression of membrane receptors (TLR2, TLR9) involved in the reduction of pathogen-induced inflammation. Like, *Lactobacillus jensenii* TL2937 in porcine intestinal epithelial cells decreases the expression of TLRs by increasing the negative regulators [IRAK-M, BCL3, toll interacting protein (TOLLIP), and A20] of these receptors and reduces the *E. coli* induced inflammation (133). Another study also reported similar anti-inflammatory effects of other probiotics (*Lactobacillus amylovorus* DSM 1669 and *L. delbrueckii* TUA 4408), including inhibition of ETEC-induced activation of the NF-κB and MAPK pathways *via* negative regulation of TLRs, which results in a decrease of pro-inflammatory cytokines (IL1, IL6, IL-1β, and IL8) and an increase of anti-inflammatory cytokine (IL10) in pig explant, caco-2, and porcine intestinal epithelial cells (134, 135). Amdekar et al. also demonstrated that *Lactobacillus* species play a key protective role against *E. coli*-induced urinary tract infection, and clearance of pathogens by regulating the expression of TLRs (TLR2 and TLR4) and subsequent production of anti-inflammatory cytokines (166). Probiotics induce the expression of different kinds of cytokines involved in host immune response during pathogenic infection by regulating the expression of TLR and their intracellular signaling pathways. They increase the expression of anti-inflammatory cytokines and reduce the inflammatory response of host cells during infection. *L. amylovorus* shows protective and anti-inflammatory effects in pig explants and caco-2 cells against *E. coli* infection and decreases *E. coli*-mediated inflammation by increasing the levels of TLR4 negative regulators (IRAK-M and TOLLIP) and decreasing those of extracellular heat shock proteins (HSP90 and HSP72), which are crucial for TLR4 functioning. This effect leads to inhibition of the *E. coli*-induced increase in the levels of TLR4 and MyD88, phosphorylation of IκBα, IκB kinase α (IKKα), IKKβ, and NF-κB subunit p65, as well as the overproduction of inflammatory cytokines (IL8 and IL1β) (134). Treatment with *L. rhamnosus* ATCC 7469 decreases TLR4 and NOD2 mRNA expression during ETEC infection in IPEC-J2 cell model and reduces the associated inflammatory response of the host. Notably, ETEC induced higher mRNA expression of these membrane

and cytoplasmic receptors that lead to the transcription of inflammatory genes *via* the NF-κB pathway (136).

Some probiotics improve the immune status of aging mice to increase their resistance against infection. The study of Sharma et al. on mice reported that *L. rhamnosus* MTCC 5897 feeding alleviates the imbalance of Th1/Th2 immune response and also increases the activity of antioxidant enzymes (catalase, glutathione peroxidase, and superoxide dismutase) and reduces *E. coli* load in the liver, spleen, and intestines by increasing the level of *E. coli* specific antibodies (IgA and IgG) (167). Similarly, *L. fermentum* MTCC 5898 feeding in aged mice increases their protection against *E. coli* infection by increasing the IgA and IgG1 levels and inflammatory proteins and reduces the *E. coli* load in the intestines, liver, spleen, and peritoneal fluids (168). Other lactobacilli improve the *E. coli* disturbed intestinal barrier function as, *E. coli* significantly decreases the intestinal permeability by decreasing the level of tight junction proteins (Occludin, ZO-1, cingulin-1, claudin-1, etc.) as observed by Bhat et al. in Caco-2 cells (169). *L. rhamnosus* (LR: MTCC-5897) improves these tight junction proteins and significantly reduces the *E. coli* induced hyperpermeability in Caco-2 cells (170). Similar effects were also observed by *L. fermentum* (LF: MTCC-5898) treatment during *E. coli* infection in Caco-2 cells in which *L. fermentum* (LF: MTCC-5898) improves the barrier integrity by reducing *E. coli* induced lower mRNA expression of Occludin, ZO-1, cingulin-1, and claudin-1 (57).

Thus, probiotics positively regulate the immune response of host cells at various steps through different mechanisms of action and protect the host from ETEC-induced deleterious effects.

Clostridial Infection

Clostridial species are rod-shaped, Gram-positive toxins and spore-producing bacteria. *Clostridium difficile* is linked to a wide range of clinical problems (171) and produces many toxins (e.g., cytotoxins and enterotoxins), which cause diarrhea (172). It mainly produces the exotoxins TcdA and TcdB with a size of ∼300 kDa. When it binds apically with epithelial gut cells, TcdA causes tight junction interruption and also facilitates the binding of TcdB toxins to the basal lamina. TcdB causes an increase in vascular permeability, release of neurotensin, induction of pro-inflammatory cytokines, fluid secretion, and eventually diarrhea (173).

Probiotics may subside the detrimental effects of clostridial infection by modulating the innate (mucus, lysozymes, and alpha defensin production, and modulation of membrane receptors such as TLRs and NLRs) and adaptive (production of immunoglobulins, anti-inflammatory cytokines, antigen uptake, and modulation of antigen-presenting cells) immune responses and cell signaling pathways (NF-κB and MAPK) of the host (173, 174). *S. boulardii* is a type of yeast that may be used as a probiotic against clostridial toxins. It increases the production of antibodies (IgA, IgG, and IgM) acting as adjuvant in BALB/c mice (121) and has numerous other mechanisms of action associated with immune regulation. It inhibits the activation of the NF-κB and MAPK signaling pathways, and pro-inflammatory cytokine (IL8) production induced by *C. difficile* toxin A in human colonic epithelial cells (NCM460). This toxin activates the extracellular signal-regulated kinase 1/2 (ERK1/2) and stress-activated protein kinases (SAPK)/Jun amino-terminal kinases (JNK) (JNK/SAPK) pathways, resulting in the transcription of pro-inflammatory cytokine (IL8) genes and leading to inflammation. *S. boulardii* inhibits the *Clostridium* toxins A-induced ERK1/2 and JNK/SAPK signaling pathways in mice (122). Furthermore, it degrades *C. difficile* toxins by its protease action and decreases the binding of toxins to host cell (rat ileum) receptors (123).

Staphylococcus Infection

Staphylococcus is a major cause of bovine contagious mastitis and persistent infection in bovine mammary epithelial cells in animals. *Via* upregulation of TLR2 and TLR4, *Staphylococcus aureus* (*S. aureus*) increases the secretion of basic fibroblast growth factor and TGF-β1 through activation of the NF-κB pathway by inhibiting NF-κB inhibitors in bovine mammary epithelial cells (175). Many probiotics are used to treat and control *Staphylococcus* infection. Probiotic *L. casei* (BL23) significantly reduces inflammation of the mammary glands during *S. aureus* infection by suppressing the expression of *S. aureus*-induced pro-inflammatory cytokines (IL8, IL6, TNF-α, IL1β, and IL1α). This results in potent anti-inflammatory effects against *S. aureus* infection in bovine mammary epithelial cells (124). *Bacillus subtilis* has shown protective effects against *S. aureus* infection in mice, by activating macrophages, limiting systemic inflammation induced by *S. aureus*, and decreasing the pathogen load. *Bacillus subtilis*-secreted exopolysaccharides (EPS) have an immunomodulatory function, producing hybrid macrophages (having the functions of both M1 and M2) with anti-inflammatory and bactericidal phagocytic characteristics. These hybrid macrophages limit *S. aureus*—induced T-cell activation and kill *S. aureus* by increasing the levels of reactive oxygen species and decreasing the levels of pro-inflammatory cytokines and chemokines [chemokine (C-C motif) ligand 2 (CCL2), CCL3, CCL4, TNF] (125). Paynich et al. (176) study on mice showed that *Bacillus subtilis*-exopolysaccharides induces anti-inflammatory macrophages (M2), which inhibit T-cell ($CD4^+$ and $CD8^+$) activation by secreting TGF-β and PD-L1 molecules. These molecules have inhibitory effects on $CD4^+$ and $CD8^+$ cells, showing a significant anti-inflammatory property in T cell-dependent immune reaction (176). In this way, probiotics beneficially regulate the immune response of host cells; they activate immune cells to kill *S. aureus* and decrease pathogen-associated inflammation by limiting the overexpression of inflammatory cytokines from pathogen-activated immune cells.

Listeria Monocytogenes Infection

Listeria monocytogenes causes several infections, including maternal-fetal infection, septicemic pneumonia, pleural infection (177), foodborne diseases with a 20–30% mortality rate (178), and neurolisteriosis leading to meningitis and encephalitis (179). Several probiotics (mostly *Lactobacilli* species) are used to protect the host against *L. monocytogenes* infection. *L. salivarius* (BGHO1) therapies against *L. monocytogenes* exert protective effects by modulating the adaptive and innate immune responses during *L. monocytogenes* infection in rats. BGHO1

increases the mRNA expression of CD14, TNF-α, and IL1β and decreases listeriolysin (*Listeria* toxins) in the intestinal tissues. In mesenteric lymph nodes, BGHO1 co-administered with *L. monocytogenes* enhances CD69 and OX-62 mRNA expression (126). *L. delbrueckii* induces the production of TNF-α and IFN-γ, which stimulates the macrophages to kill *L. monocytogenes*. Mice infected with *L. monocytogenes* which received *L. delbrueckii* UFV-H2b20 have a longer lifespan, less liver immunopathology, and less bacterial load in the spleen and liver (127). These probiotics stimulate macrophages by inducing the expression of specific cytokines to increase their bactericidal activities and decrease the level of toxins, as well as assist the host in eliminating pathogens from their body and accelerate recovery.

Heat-killed *E. faecium* BGPAS1-3 cell wall protein, which is resistant to high temperature, has shown protective and strong anti-listeria activity. It stimulates Caco-2 cells to increase TGF-β production. TGF-β exerts protective effects on epithelial tight junctions by upregulating the expression of claudin (128). These innate immunomodulatory effects are achieved by modulation of the MyD88-dependent TLR2 and TLR4 pathways in intestinal cells against *Listeria* infection. *L. monocytogenes* induces TLR2 and suppresses the expression of TLR4 mRNA in Caco-2 cells. Heat-killed BGPAS1-3 decreases the expression of TLR2 mRNA in Caco-2 cells. In contrast, the expression of TLR4 mRNA in Caco-2 cells is increased by both heat-killed and live BGPAS1-3 before and after *L. monocytogenes* infection, respectively. Furthermore, heat-killed or live BGPAS1-3 has inhibitory effects on the expression of IL8 in uninfected and infected *L. monocytogenes* Caco-2 cells (180). Heat-killed and live probiotics, as well as their cellular components, can regulate the immune response of the host through interaction with TLRs, increase the protective innate immune response, and decrease the inflammatory response of host cells. Cho et al. showed the protective and immunomodulatory effects of heat-killed and live *E. faecium* JWS 833 using a *L. monocytogenes* mice model and peritoneal mouse macrophages, respectively. Both heat-killed and live JWS833 show immunomodulatory properties. When administered orally, live JWS833 increases the levels of serum cytokines (TNF-α and IL1β) and NO against *L. monocytogenes* in mice. Heat-killed JWS833 stimulates the macrophages to produce TNF-α, NO, and IL1β (129). Probiotics have diverse immunomodulatory functions, assisting the host to counter pathogenic infections.

Viral Diseases and Probiotics

The threat of viral illness has recently increased significantly due to the changes in the environment (e.g., anthropogenic climate change and increased global movement of passengers and cargo). Viral infections cause variable morbidity and mortality with a detrimental effect on community well-being and cause widespread economic losses. Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), which infected millions of people worldwide during the 2019–2020 pandemic is a good example of this global economic loss (181). Thus, finding alternative and effective strategies to prevent viral infections and reducing the morbidity and mortality of viral infections is critical (**Table 3**). Nevertheless, many vaccines and antiviral drugs aiming to be effective in infections are available, but a major challenge is the new viral strains that appeared after mutations, particularly in RNA viruses. It is wise to have some alternative strategies that could be used as supplemental or preventive remedies. To reduce the severity of viral infections and their numbers, a balanced diet including nutrients or food additives that boost and potentiate immune system response, is a beneficial alternative measure. The use of probiotics is one of the dietary approaches used in recent years to increase immunity and decrease the risk of infections (213). Many probiotics (mainly *Lactobacilli* species) are used for the prevention or treatment of viral illnesses. In addition, to alter the crosstalk between gut bacteria and the mucosal immune system, probiotics have many other immune modulatory and non-immune functions to combat viral incursion. The application of probiotics for the control and prevention of clinically important viral diseases is briefly discussed below.

Rotavirus

Bifidobacterium infantis (MCC12) and *Bifidobacterium breve* (MCC1274) modulate immune response during human rotavirus infection in the porcine intestinal epithelial cell line. Both species are able to blunt IL8 production and increase IFN production by increasing the activation of interferon regulatory factor 3 (IRF3) through the suppression of A20 (a zinc-finger protein with negative effects on IRF3 activation) (182). These probiotics activate various interferon-stimulated genes (ISGs), including RNase L ($2^{'}$-$5^{'}$ oligoadenuylate dependent endoribonulecase) and myxovirus resistance protein A (MxA) (183). MxA decreases virus replication by binding with virus nucleoproteins (219). RNase L has antiviral activity and lessens viral replication through the elimination of infected cells by inducing apoptosis and IFN amplification by activating RLRs (220, 221). RLRs are intracellular PRRs involved in virus recognition. *L. rhamnosus* GG (strain ATCC 53103) and *B. lactis* Bb12 enhance the efficacy of human attenuated rotavirus vaccine (AttHRV) during rotavirus infection in gnotobiotic human rotavirus pig model, by increasing T-cells subset ($CD3^{+}$, $CD4^{+}$) in intestinal tissues and T-cells subset ($CD3^{+}$, $CD8^{+)}$ in the blood and spleen. Further, the severity of diarrhea and virus load was also less in vaccinated pigs receiving ATCC 53103 and Bb12 as compared to only vaccinated pigs (184). Similarly, *S. boulardii* and several *Bifidobacterium* and *Lactobacillus* species have anti-rotaviral effects, mitigate the severity and duration of diarrhea, viral shedding, and incidence of infections associated with rotavirus, and modulate the immune response of the host (222–226) *Lactobacillus* species and *Bifidobacterium* in combination with some prebiotics (human milk oligosaccharide, short-chain galactooligosaccharides, and long-chain fructooligosaccharides) show antiviral response. *L. casei* (Lafti L26-DSL) and *Bifidobacterium adolescentis* (DSM 20083) reduced the infectivity of virus in MA104 cells (embryonic Rhesus monkey kidney cells) by interacting with virus protein (NSP4). NSP4 has been characterized as virus toxin and is associated with diarrhea in host (185, 186). *L. rhamnosus* (strain GG) and Gram-negative *E. coli* Nissle (*EcN*) decrease human rotaviral complications by modulating the immune

TABLE 3 | Probiotics therapies during viral diseases.

Probiotics	Target viruses	Study models	Mechanism of action	Effects	References
Bifidobacterium infantis (MCC12)	Rotavirus	PIE cells	↓ IL-8, ↓ A20, ↑ IRF3, ↑ IFN, ↑ ISGs	↓ Virus replication ↑ Infected cells apoptosis	(182, 183)
Bifidobacterium breve (MCC1274)	Rotavirus	PIE cells	↓ IL-8, ↓ A20, ↑ IRF3, ↑ IFN	↓ Virus replication ↑ Infected cells apoptosis	(182, 183)
Bifidobacterium lactis Bb12	Rotavirus	Pig rotavirus model	↑ T cells subset ($CD3^+$, $CD4^+$) ↑ Vaccine efficacy	↓ Virus load	(184)
Bifidobacterium adolescentis (DSM 20083)	Rotavirus	MA104 cells	Interact with virus protein (NSP4)	↓ Diarrhea	(185, 186)
L. rhamnosus GG (strain ATCC 53103)	Rotavirus	Pig rotavirus model	↑ T cells subset ($CD3^+$, $CD4^+$) ↑ Vaccine efficacy	↓ Virus load	(184)
L. casei (Lafti L26-DSL)	Rotavirus	MA104 cells	Interact with virus protein (NSP4)	↓ Diarrhea	(185, 186)
L. acidophilus and *L. reuteri*	Rotavirus	Pig model	↑ Intestinal IgM and IgG ↑ Serum IgM titers ↑ Total intestinal IgA secreting cell response	↓ Virus load	(187)
Lactobacillus delbrueckii ssp. bulgaricus OLL1073R-1 fermented yogurt	Influenza virus	96 volunteers	Affect IgA levels in saliva	Help to prevent influenza infection	(188)
L. paracasei	Influenza virus	Mice	↑ IL1α and IL1β before infection ↑ Recruite immune cells before infection ↑ IL10 after infection	↓ Viral load ↓ Morbidity ↓ Mortality	(189)
L. casei DK128	Influenza virus	Mice	↑ IgG1, IgG2a, ↓ IL6 and TNF-α ↑ Monocytes	↓ Inflammation ↑ Host survival rate	(190)
L. plantarum (O6CC2)	Influenza virus	Mice	↑ IFN-a and Th1 cytokines	↓ Infection severity	(191, 192)
L. paracasei CNCM I-1518	Influenza viruses	Mice	↑ Early recruitment of IL-1α, IL-1β Recruit immune cells before infection	↑ Protection against virus	(189)
L. plantarum (AYA)	Influenza virus	Mice	↑ IgA	↓ Infection severity	(193)
L. GG and *L. johnsonii* (NCC 533)	Influenza virus	Mice	↑ IgA, IFN-g	↓ Mortality ↓ Morbidity ↓ Virus titer ↓ Cell death	(194)
Bifidobacterium longum BB536	Influenza virus	Mice	↑ Activities of neutrophils and NK cells.	↓ Weight loss ↓ Virus replication ↓ Infection severity	(195, 196)
L. plantarum (137)	Influenza virus	Mice	↑ IFN-β	↓ Infection severity	(197)
L. delbrueckii ssp. bulgaricus OLL1073R-1 fermented yogurt	Influenza virus	96 volunteers	Affect IgA levels in saliva	Help to prevent influenza infection	(188)
L. acidophilus NCFM and *Bifidobacterium animalis* subsp. *lactis* Bi-07	Influenza virus like symptoms	326 children	–	↓ Fever incidence (53.0%) ↓ Coughing incidence (41.4%) ↓ Rhinorrhea incidence (28.2%)	(198)
Recombinant *L. plantarum*	Corona viruses (TGEV and PEDV)	IPEC-J2	↑ ISGs (OASL, ISG15, Mx1) ↑ B^+IgA^+, IgG ↑ IFN-γ	↓ Infection severity	(199, 200)
L. casei ATCC39392 vaccine	TGEV	Pig model	↑ Antibodies ↑ IL17	↓ Infection severity	(201)
L. plantarum Probio-38 and *L. salivarius* Probio-37	TGEV	ST cell line	Inhibit virus	↓ Infection severity	(202)
cell-free supernatants of *L. plantarum* 22F, 25F, and 31F, live *L. plantarum* (22F, 25F)	PEDV	Vero cells	Antiviral activity	↓ Infection severity	(203)
Mixture of different Lactobacilli and Bifidobacteria	HIV	Clinical trial on 8 human positive patients	↑ Serotonin in blood ↓ Tryptophan in plasma		(204)
L. rhamnsosus GR-1 and *L. reuteri* RC-14	HIV	Clinical trial of 65 confirmed women	–	Improved life quality of women	(205)

(Continued)

TABLE 3 | Continued

Probiotics	Target viruses	Study models	Mechanism of action	Effects	References
L. plantarum 299v	HIV	Clinical trial of 14 children	Stabilize $CD4^+$ T cells numbers	↓ Inflammation	(206)
S. boulardii CNCM I-745	HSV-1	Mice	↑ Anti-inflammatory cytokines ↓ pro-inflammatory cytokines	↓ Gastrointestinal dysfunctioning	(207)
L. rhamnosus BMX 54	Human papillomavirus (HPV)	Clinical trial of 117 women	–	Favors recreation of vaginal balance, may be useful to control HPV infection	(208)
Bifidobacterium bifidum	HPV	Mice	↑ IL2 ↑ IFN-γ	↓ Virus complication, prevent tumor growth	(209)
L. reuteri RC-14 and *L. rhamnosus* GR-1	HPV	Clinical trial of 180 women	–	↓ Abnormal cervical smear rate, no effect on virus clearance	(210)
L. rhamnosus PTCC 1637 and *E. coli* PTCC 25923	Herpes simplex virus-1	African green monkey kidney cells	↑ Viability of macrophages Competitive adhesion with cells	↑ Virus elimination Antiviral effects	(211)
Enterococcus faecalis FK-23	Hepatitis C virus	*In vitro* trial of 39 positive patients	↓ Alanine transferase	Improve health	(212)
Bifidobacterium bifidum 2-2, *Bifidobacterium*. *bifidum* 3-9, *L. gasseri* TMC0356, *L. casei* TMC0409, *L. rhamnosus* LA-2 *L. rhamnosus* (LGG), Streptococcus thermophilus TMC1543	Enteric common infectious diseases	Bovine intestinal epithelial cell line	↑ TLR3 activation ↑ IFN β	↑ Protection against enteric viruses	(213)
L. fermentum PCC, *L. casei* 431 and *L. paracasei*	Upper respiratory tract viruses and influenza viruses	Clinical trial of 136 volunteers	↑ Serum IFN-γ ↑ Intestinal IgA	↓ Symptoms of flue and respiratory tract infection incidence	(214)
L. plantarum DR7	Upper respiratory tract virus's infection	Clinical trial of 209 adults	↑ IL-4, IL-10, CD44, CD117 ↓ IFN-γ, TNFα, CD4, CD8	↓ Nasal symptoms and frequency of URTI ↓ Oxidative stress ↓ Plasma peroxidation	(215)
Bifidobacterium bifidum G9-1 (BBG9-1)	Rotavirus	BALB/c mice	Induced mucosal protective factors	Improve lesion and diarrhea	(216)
L. helveticus R0052 and *L. rhamnosus* R0011	Rotavirus, Adenovirus, Norovirus	Clinical trial of children (816)	–	No beneficial effects	(217)
L. paracasei N1115	Upper respiratory tract viruses	274 clinical volunteers' trial	May stimulate T cell immunity	Protection against acute respiratory tract infection	(218)

response and interacting with rotavirus. In the pig rotavirus model, *EcN* and *L. rhamnosus* GG induced higher total IgA levels in the intestine and serum post- and pre-human rotavirus challenge, respectively, and reduced viral shedding. *EcN* can regulate the expression of cytokines (IL6 and IL10) and bind with rotavirus protein 4 to reduce rotavirus attachment to the host cells (227, 228). In the rotavirus gnotobiotic pig model, *Lactobacilli* species (*L. acidophilus* and *L. reuteri*) significantly increased total intestinal IgM and IgG and serum IgM titers and total intestinal IgA secreting cell responses (187). Furthermore, Azevedo et al. (229) demonstrated that these probiotics (*L. acidophilus* and *L. reuteri*) significantly increased Th1 and Th2 cytokines in human rotavirus infected pigs, and also help in maintaining immunological homeostasis during human rotavirus infection by regulating the production of TGF-β. Different probiotics have anti-rotavirus activities involving various immunomodulatory mechanisms. *Bifidobacterium* stimulates ISGs and lowers various pro-inflammatory cytokines, while *Lactobacillu*s increases anti-rotavirus antibodies and reduces rotavirus-associated complications.

Influenza Virus

A randomized controlled trial on 96 elderly people showed that a yogurt fermented with *L. delbrueckii ssp. bulgaricus* OLL1073R-1 (1073R-1-yogurt) affected the level of influenza A H3N2 bound IgA levels in saliva (188). *L. acidophilus* NCFM and *Bifidobacterium animalis* subsp. *lactis* Bi-07 reduce the incidence of coughing (41.4%), rhinorrhea (28.2%), and fever (53%) in a double blind placebo controlled study on 326 children during the winter season (198). Different clinical trial studies on children, elderly people, adults, and animals compiled by Lehtoranta et al. (230) shows that probiotic administration reduced the risk respiratory viruses including influenza viruses. In mice, *L. paracasei* showed anti-influenza effects and beneficially modulated the immune response against influenza infection, while reducing the viral load, morbidity, and mortality. *L. paracasei* increases the levels of pro-inflammatory cytokines (IL1α and IL1β) and recruitment of immune cells before infection. This accelerates viral clearance and reduces the levels of inflammatory cytokines [macrophage inflammatory protein-1α (MIP1α), IFN-γ, MCP-1, and MIP1β] after influenza

infection. Moreover, *L. paracasei* has shown anti-inflammatory characteristics at the late stage of infection by increasing the levels of IL10 (189). Heat-killed *L. casei* DK128 shows similar anti-inflammatory effects against influenza infection by decreasing influenza virus-induced pro-inflammatory cytokines (IL6 and TNF-α), monocytes, and activated NK cells in the lungs of mice, thereby preventing pulmonary inflammation. Furthermore, DK128 increases the levels of antibodies (IgG1 and IgG2a) against the influenza virus at an earlier time point and provides cross-immunity against secondary heterosubtypic influenza infection with improved health and survival rate in mice (190). *L. plantarum* (O6CC2) beneficially modulates the host immune response during influenza infection in mice by increasing the production of IFN-α and Th1 cytokines (IL12 and IFN-γ) as well as the expression of Th1 cytokine receptors which potentiate NK cell activity at the early stage of influenza infection in mice. Of note, NK cells are an important line of defense during this early phase (191, 192). At the late stage of infection, *L. plantarum* (O6CC2) decreases IL6 and TNF-α production to control influenza-mediated inflammation. Furthermore, O6CC2 decreases neutrophil and macrophage infiltration to overcome the inflammatory response to influenza infection (231). *L. plantarum* (AYA) has shown protective immunological effects against influenza virus infection by increasing production of mouse mucosal IgA (193). *L. GG* and *L. johnsonii* (NCC 533) are also associated with increased IgA production (232, 233). *B. longum* (MM-2) has shown anti-influenza activity by enhancing the innate immunity through increases in the expression of NK cell activator genes (IFN-g, IL2, IL12, IL18) activities. This probiotic reduces mortality, morbidity, virus titer, cell death, virus-induced inflammation, and the expression of mRNA for pro-inflammatory cytokines (IL6, TNF-α, IL1β, MIP2, and MCP-1) in mice infected with influenza virus (194). Similar immune regulatory and anti-influenza effects of *Bifidobacterium* have been observed by other researchers. *B. longum* BB536 enhances the activities of neutrophils and NK cells, reduces fever in human beings (195), reduces IL6 and IFN-γ at the late stage of infection, and prevents body weight loss and virus replication in the lungs of mice infected with the influenza virus (196). *L. plantarum* (137) induces higher type-1 interferon (IFN-β) levels in the serum of mice at the early stage of influenza infection (197). Notably, innate immunity of type-1 interferon is involved in countering viral infection at the early stage (234). In the case of the influenza virus infection, gut microbiota have preventive effects and modulate type I IFNs (235). These IFNs are involved in innate immunity during viral infection with antiviral activities, as well as the degradation and inhibition of viral nucleic acids and viral gene expression, respectively (236, 237). These studies showed that various probiotics show anti-influenza activities along with immunoregulatory effects during infection.

Coronavirus

Coronavirus disease 2019 (COVID-19) was officially declared as a pandemic by WHO on March 11, 2020 (238). SARS-CoV-2 was first identified in Wuhan city (China) in December 2019 (239) in patients with pneumonia and rapidly spread to 216 countries (240, 241). Coronaviruses (CoVs) belong to the family Coronaviridae and genus coronavirus order Nidovirales and subfamilies: Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus (242). Subfamilies alphacoronavirus and Betacoronavirus originate from mammals mainly bats, and Gammacoronavirus and Deltacoronavirus subfamilies originate from pigs and birds (243). In SARS-CoVs virion envelop, there are three main structural proteins—protein S (Spike), protein M (membrane), and protein E (envelop). Protein S (Spike) facilitates the SARS-CoVs adherence and fusion (52). All CoVs are positive sense, single stranded RNA, and pleomorphic viruses with typical crown shape peplomers of 27–32 kb and 80–160 nM size (239, 244). Genomic structure analysis showed that the viruses belong to β-coronavirus including MERS-CoV and SARS-CoV with high mutation rates because of RNA dependent DNA polymerase transcription error (242), which is also the main target of drug discovery (245). Pathogenesis of SARS-CoV-2 includes binding of its spikes proteins (S) to Angiotensin-Converting-Enzyme-2, which are highly expressed in lungs as well as in esophagus and enterocytes in the colon and ileum, to get entry into the cells for infection (246). TMPRSS2 is a protein, which helps the "S" proteins of SARS-CoV-2 to get entry into cells, is also highly expressed in absorbent enterocytes (247). Clinical signs of COVID-19 disease are different ranging from asymptomatic to non-specific flu and severe pneumonia, Middle Eastern respiratory syndrome (MERS) (248), and life-threatening consequences like acute respiratory distress syndrome and different organ failure. It can also affect neurological, gastrointestinal, and hepatic systems (249). According to data from Wuhan city in China, 14% of the infected cases were severe, 4% died, and 5% needed intensive care (250).

In spite of the different measurements including hygienic improvement, screening, and social distancing, COVID-19 is rapidly spreading and progressing worldwide (22, 240), while the search for effective drugs and vaccine therapies is underway. Scientists are battling against the time needed to develop a vaccine, but it is hard to make an efficient and safe product as rapidly as the virus is spreading (251). Thus far, there are no effective drugs available for SARS-CoV-2. However, according to genomic structure analysis and its similarity with SARS and MERS, certain drugs (e.g., lopinavir, ritonavir, and nitazoxanide) may be applicable (252). At the same time, several studies have compiled alternative data related to general viruses management and treatment (253–258) including nutritional supplements like vitamins and some other immune boosts medicine (259). Some *in silico* data are in favor of probiotics use for the treatment of COVID 19 as data indicate that probiotics derived molecules like lactococcin Gb (*L. lactis*), subtilisin (Bacillus amyloliquefaciens), sakacin P (*L. sakei*) may inactivate "S" glycoprotein and its receptors molecules i.e., Angiotensin-Converting-Enzyme-2 (260). Similarly, several other studies have published their data regarding the use of probiotics for the general management of viral diseases as it is indicated by some clinical evidence that some kinds of probiotics are helpful in preventing bacterial and viral infections like respiratory tract infections, sepsis, and gastroenteritis. Viruses account for over 90% of upper RTIs as etiological agents. Many studies have

recorded the positive effect of probiotics on the protection of upper respiratory tract infections. Reduced risk of getting upper respiratory tract infections in probiotic supplementations was recorded in a meta-analysis study of 12 randomized control trials involving 3,720 children and adults. It was observed in 479 adults of a randomized, double-blind, placebo-controlled intervention study that *B. bifidum* MF 20/5, *L. gasseri* PA 16/8, and *B. longum* SP 07/3 along with mineral and vitamins reduced the duration of fever and common cold (22). *Streptococcus salivarius* strain K12 may possibly reduce the severity of COVID-19 complications by its ability to maintain stable upper respiratory tract microbiota. As advanced studies have shown that lung microbiota have an important role in the homeostasis of immune responses (261), and its dysbiosis makes the patient more vulnerable to viral infections. In the case of COVID-19, a significant difference in lung microbiota has been observed in patients with COVID-19 and normal persons (262). Probiotic consumption triggers pro- and anti-inflammatory cytokines production to clear the viral infection, reduce the cell damage in the lungs, and improve the levels of T cells, B cells, NK cells, and type 1 interferons in the immune system of the lungs, and it may help to prevent COVID-19 complications (261).

Probiotics and recombinant probiotics with antiviral effects are effectively used to combat and minimize the detrimental effects of other coronaviruses, such as alphacoronaviruses—particularly transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV)—which cause substantial economic losses in the pork meat industry. Recombinant *L. plantarum* inhibits TGEV and PEDV infections in the IPEC-J2 cell line by enhancing ISGs (OASL, ISG15, and Mx1) which have strong antiviral effects (199). Recombinant *L. plantarum* (containing the surface S antigen of TGEV) elicits an immune response characterized by higher numbers of activated DC cells, $B^{+}IgA^{+}$ cells, secretory IgA (sIgA), serum IgG, IFN-γ, and IL4 which help the host to combat TGEV (200). Similar effects were observed by Jiang and colleague who reported that a recombinant *L. casei* ATCC39392 vaccine modulates the immune response against TGEV infection, induces IL4, mucosal (IgA), and systemic (Ghosh and Higgins) antibodies, and polarized Th2 immune response with enhanced the expression of IL17 against TGEV in a pig model (201). Similarly, immune protective effects with the elicitation of sIgA and IgG production against PEDV have also been shown by a *L. casei*-based vaccine, consisting of a DC-targeting peptide attached to the PEDV core antigen (263). Antibiotics and porcine bile-resistant *L. plantarum* Probio-38 and *L. salivarius* Probio-37 have shown antiviral effects *in vitro* ST cell line and inhibit TGE coronavirus without cytotoxic effects (202). Another study shows that cell-free supernatants of different LAB (*L. plantarum* 22F, 25F, and 31F) and live *L. plantarum* (22F, 25F) have anti PEDV activity with any cytotoxic effects on Vero cells (203). *E. faecium* has protective effects against enteropathogenic coronavirus TGEV and hinders the virus entry into cells by interacting with cell surface molecules, reducing viral structural proteins, and inducing antiviral NO (264, 265). Furthermore, *E. faecium* stimulates an antiviral response by increasing the expression of IL8 and IL6 mRNA (266), which contribute to the immune regulation against many other enteric pathogens (267). Studies show that *E. faecium* (probio-63) and *E. faecalis* (KCTC 10700BP) suppress coronavirus growth, responsible for porcine epidemic diarrhea (268, 269). These findings indicate that probiotics have antiviral effects, and stimulate the immune response of the host against viruses. Many probiotics enhance vaccine efficacy; some probiotics inhibit virus entry into cells and also stimulate the production of different cytokines during viral infection.

Probiotics and Parasitic Diseases

Probiotics are widely applicable to the treatment and prevention of parasitic infections (**Table 4**). Oral administration of *L. rhamnosus* MTCC 1423 during Giardia infection in mice modulates both cellular and humoral immune responses, enhances sIgA, IgA^{+} cells, $CD4^{+}$ T lymphocytes, and anti-inflammatory cytokine IL10, and decreases pro-inflammatory cytokine IFN-γ (277). *E. faecium* SF 68 stimulates an anti-giardia immune response, increases $CD4^{+}$ T cells and the production of anti-giardia antibodies (intestinal IgA and serum IgG), and reduces the parasitic load (278). *Lactobacillus* and *S. boulardii* also have positive effects in the treatment of giardiasis, minimizing interaction between the host and pathogen, reducing parasite load, and modulating the immune response of the host. *L. johnsonii* La1 (NCC533) reduces active trophozoite of Giardia intestinalis strain WB and infection duration in *Meriones unguiculatus* (286). Recombinant *L. plantarum* NC8 (containing *Eimeria tenella* protein) induced a higher percentage of a T-cell subset ($CD3^{+}$, $CD4^{+}$, and $CD8^{+}$) and antibody levels, provided protection against *E. tenella* infection in chickens, and reduced lesion, cecum damage, and oocyst shedding (270). *L. salivarius*, *L. johnsonii*, and *S. cerevisiae* provided protection against *Eimeria* infection in chickens; reduced oocyst count, improved weight gain and FCR, and stimulated the immune response with higher antibodies (IgM and IgG) titer and lymphoproliferative response (271). Pender et al. revealed that chickens receiving supplementation of commercially available probiotics; Primalac W/S (*L. acidophilus*, *L. casei*, *E. faecium*, and *B. bifidium*) showed lower mortality, higher body weight, and fewer *Eimeria maxima*-, *Eimeria tenella*-, and *Eimeria acervulina*-induced lesions; however, there was no effect on the immune response (272). Lactic acid from *L. acidophillus* stimulates the host immune response during *Cryptosporidium* infection, increasing the number of lymphocytes, levels of complement proteins (C3, C4), and antibodies (IgM, IgG), as well as reducing oocyst shedding from infected rabbits (273). *L. casei*, Bifidium bacteria, and *E. faecalis* exert protective effects during *Cryptosporidium parvum* infection and greatly reduce parasite load and oocyst shedding from the intestine of infected mice (287–289). In contrast, Oliveira and Widmer demonstrated that some commercially available probiotics enhanced the severity of cryptosporidia infection by altering the intestinal environment in favor of *C. parvum* proliferation (290). *Bifidobacterium animalis subspecies lactis* strain Bb12 stimulates local immune response during *Ascaris suum* infection in juvenile pigs and production of anti-parasite antibodies (IgA in serum and IgG1 and IgG2 in ileal fluid) and glucose uptake

TABLE 4 | Probiotics therapies during parasitic diseases.

Probiotics	Parasites	Study models	Mechanism of action	Effects	References
Recombinant *L. plantarum* NC8	*Eimeria tenella*	Chicken	↑ $CD3^+$, $CD4^+$, $CD8^+$ ↑ IgA, IgM and IgG	↓ Lesion ↓ Cecum damage ↓ Oocyst shedding ↓ Inflammation	(270)
L. salivarius, *L. johnsonii*, and *S. cerevisiae*	*Eimeria tenella, Eimeria maxima, Eimeria necatrix*	Chicken	–	↓ Oocyst count ↑ Weight gain ↑ FCR	(271)
Primalac W/S (*L. acidophilus*, *L. casei*, *Enterococcus faecium*, and *Bifidobacterium bifidium*)	*Eimeria maxima*, *Eimeria tenella*, and *Eimeria acervulina*	Chicken	–	↓ Lesion	(272)
L. acidophillus lactic acid	*Cryptosporidium parvum* oocysts	Rabbit	↑ Complement proteins (C3, C4) ↑ Lymphocytes ↑ IgM and IgG	↓ Parasitic load	(273)
Bifidobacterium animalis	*Ascaris suum*	Juvenile pigs	↑ IgA in serum ↑ IgG1 and IgG2 in ileal fluid	↓ Parasitic complications	(274)
L. rhamnosus	*Ascaris suum*	Pigs	↑ TLR9 expression ↑ TNF-α, IFN-γ, and IL10	↓ Parasitic allergic complications	(275, 276)
L. rhamnosus	*Giardia intestinalis* (Portland strain I)	BALB/c mice	↑ sIgA, IgA^+ cells, $CD4^+$ ↑ T lymphocytes ↑ IL10 ↓ IFN-γ	↓ Giardia infection severity Restore intestinal morphology	(277)
Enterococcus faecium SF68	*Giardia intestinalis* H7 (ATCC 50581)	Mice	↑ Intestinal IgA ↑ Serum IgG ↑ $CD4^+$ T cells	↓ Parasitic load	(278)
L. plantarum, *L. reuteri*, *L. casei*, and *L. acidophilus*	*Schistosoma mansoni*	Mice	↑ IgM ↓ AST, LDH, and gGT	↓ Parasitic complications ↓ Spleen and liver weight	(279)
L. sporogenes	*Schistosoma mansoni*	Mice	↓ Schistosomiasis cytokine-induced chromosomal aberration	↓ Chromosomal aberration	(280)
L. plantarum	*Trichinella spiralis*	Mice	↑ Serum IFN-γ	↓ Larval count ↓ Inflammation	(281)
L. fermentum, *Enterococcus faecium*, *Enterococcus durans*	*Trichinella spiralis*	Mice	↑ Phagocytic activity of leukocytes	↑ Protection	(282)
L. casei	*Trichinella spiralis*	Mice	↑ IgA and IgG	↑ Protection	(283)
L. rhamnosus (JB-1)	*Trichuris muris*	Mice	↑ IL10 ↑ Mucus-secreting goblet cells	↑ Larval removal	(284)
S. boulardii	*Toxocara canis*	Mice	↑ IL12 and IFN-γ	↑ Protection	(285)

(274). Similarly, *L. rhamnosus* modulates the expression of TNF-α, TLR9, IFN-γ, and IL10 gene, which results in decrease in eosinophil action and allergic skin reaction induced by *Ascaris suum* in the pig model (275, 276). Many probiotics are effective against schistosomiasis; *Zymomonas mobilis* stimulates immune response and provides 61% protection during schistosomiasis (291). *L. plantarum*, *L. reuteri*, *L. casei*, and *L. acidophilus* stimulate IgM antibodies against *Schistosoma mansoni* infection in mice (279). *L. sporogenes* reduces schistosomiasis cytokine-induced chromosomal aberration in mice (280). During trichinellosis (*Trichinella spiralis* infection in mice), *L. plantarum* increases the levels of IFN-γ and reduces larval count (281). *L. fermentum*, *E. faecium*, and *Enterococcus durans* enhance the activity of phagocytes during *Trichinella spiralis* infection in mice (282). *L. casei* induces IgA and IgG during *T. spiralis* infection in mice (283, 292). In trichuriasis mice model, *L. rhamnosus* (JB-1) increases IL10 and mucus-secreting goblet cells, resulting in the faster removal of larvae (284). *E. faecalis* CECT7121 (*Ef*7121) and *S. boulardii* are associated with larvicidal activity and high production of IL12 and IFN-γ, respectively, during *Toxocara canis* infection in mice (285, 293). Different probiotics have different mechanisms of action during parasitic infections. They reduce complications, regulate cytokine production, and facilitate the production of anti-parasitic antibodies. However, it has been shown that some probiotics enhance the parasitic infection as indicated in the study by Dea-Ayuela and colleague on mice in which they reported that *L. casei* decreases cytokines (IFN-γ, TNF-α, IL-4, and Il-13) and antibodies (fecal IgA) against *Trichuris muris*, increasing the susceptibility of *T. muris* infection. This *L. casei* associated increased susceptibility to infection may be related to deactivation of TNF-α dependent Th2 effector responses against *T. muris* due to the strong inhibitory effect of *L. casei* on this cytokine (294).

PROBIOTICS THERAPIES IN NON-INFECTIOUS DISORDERS

Probiotics improve the central nervous system and mental function with beneficial effects reported in anxiety, Alzheimer's disease, depression, schizophrenia, and autism (295). In an autism spectrum disorder mice model, an *L. reuteri* diet led to a behavioral improvement in an oxytocin-dependent manner (296). Probiotics can alter the composition of gut microbiota (297), which in turn acts on the gut–brain axis by secreting neuroactive substances (298) and significantly influences and regulates cerebrovascular diseases, neurodegeneration, and mental dysfunction (299). *B. infantis* reduces stress by increasing the levels of tryptophan in plasma, decreasing the levels of serotonin in the frontal cortex, and regulating the hypothalamic–pituitary–adrenal axis. *L. rhamnosus* JB-1 decreases the expression of gamma aminobutyric acid receptor and corticosterone levels in mice, which are induced during stress (300–302). Moreover, *B. longum, L. helveticus*, and *L. plantarum* reduce anxiety (303). *L. fermentum* NCIMB can produce ferulic acid, which is a strong antioxidant that can stimulate the proliferation of the nervous system stem cells and be used to treat neurodegenerative disorder, diabetes, and obesity. In mice, feeding ferulic acid ameliorates Alzheimer's disease symptoms, oxidative stress, and neuroinflammation (65). Thus, probiotics have positive effects on brain function, by affecting the functions of the nervous system as well as some related hormones and their receptors (**Table 5**). However, a detailed study of the effects of probiotics on the nervous system is needed to support the currently available evidence.

Different probiotics regulate obesity (323), which predisposes individuals to different diseases, such as non-alcoholic fatty liver diseases, cardiovascular diseases, diabetes, cancers, and some disorders related to the immune system (324). *L. plantarum* CBT LP3 and *B. breve* CBT BR3 reduce obesity related marker, and *L. rhamnosus*, *E. faecium*, *L. acidophilus*, *B. bifidum*, and *B. longum* decrease low-density lipoprotein cholesterol, total cholesterol and oxidative stress level in an *in vivo* human trial (320). *B. bifidum* W23, *B. lactis* W51&W52, *L. lactis* W19&W58, *L. brevis* W63, *L. casei* W56, *L. acidophilus* W37, and *L. salivarius* W24 regulate the obesity by decreasing triglyceride, total cholesterol, homocysteine, and TNF-α level in a randomized double-blind placebo-controlled trial on 50 women who were obese (321). Indigenous microbiota play a key role in obesity by harvesting energy for the host through different metabolic pathways. Probiotics change the composition of gut microbiota, thereby influencing obesity (12). Gut microbiota contribute to obesity *via* several potential mechanisms, such as lipogenesis, carbohydrate fermentation, and energy storage, and through numerous pathways (e.g., different hormones, metabolites, and neurotranmitters), which regulate energy balance and food intake (**Table 5**).

Probiotics also reduce the risk of cancer by different mechanisms of action, which include the exclusion of oncogenic bacteria, improvement of epithelial barrier function, increase of tumor cell death by apoptosis, production of immune-modulating metabolites (acetate, butyrate, propionate, conjugated linoleic acids, etc.), increase of cytokine production with an antitumor response, and TLR modulation. Butyrate regulates cell proliferation, differentiation, and apoptosis (325), it can stimulate anti-inflammatory cytokines and IL10 production and decrease the production of inflammatory cytokines *via* inhibition of NF-κB. Furthermore, butyrate regulates apoptosis-regulating proteins [CASP7, CASP3, BCL2 antagonist/killer (BAK), and BCL2], suppresses COX2 activity, stimulates the production of AMPs, and increases glutathione-S-transferase. These effects lead to downregulation or upregulation of genes related to the apoptosis, proliferation, and differentiation of cells (326, 327). Propionic acids and acetic acid have also shown anti-inflammatory activities by suppressing NF-κB activation and modulating the expression of pro-inflammatory genes (328). Some probiotics (*Lactobacilli*, *bifidobacteria*, and *streptococcus*) can produce conjugated linoleic acid, which has pro-apoptotic and anti-proliferative activities. This is achieved by increasing the expression of peroxisome proliferator-activated gamma receptor (PPARγ), which is involved in immune function and apoptosis. Some probiotics show their anti-cancerous activities *via* cation exchange between their peptidoglycan and the carcinogenic compound. Furthermore, probiotics decrease the COX2 enzyme-mediated production of prostaglandins, which increases the risk of colorectal cancer (329, 330). Probiotics can increase the production of immunoglobulins, such as IgA, generating an anti-inflammatory environment. IgA does not provoke activation of the complement system and acts as a barrier to reduce contact between the carcinogenic compound in the lumen and colonocytes, thereby reducing the risk of cancer (331). A prospective study involving 82,220 individuals showed that individuals who consume yogurt and sour milk are less susceptible to bladder cancer. An Italian cohort study on 45,000 volunteers of a 12-year follow up without comparative group, reports that yogurt consumption decrease in colorectal cancer (332). *L. casei* administration in humans for 4 years showed less recurrence of adenoma atypia, and probiotics with oligofructose-enriched inulin preparation reduce DNA damage in colonic epithelial cells and HT29 cells (322). Animal studies supported the beneficial effects of yogurt against genotoxic amines and cancer of the bladder and colon. In a breast cancer mice model, *L. acidophilus* isolated from yogurt promoted the proliferation of lymphocytes and decreases tumor growth (323) (**Table 5**). Hence, probiotics reduce the risk of cancer by different mechanisms. Some probiotics assist in excluding the oncogenic bacteria, while others inhibit inflammatory pathways and increase apoptosis of tumor cells. Furthermore, probiotics stimulate the production of immune-modulating metabolites involved in cell growth, proliferation, and apoptosis.

Many probiotics have beneficial effects on allergies (**Table 5**). *L. rhamnosus* (MTCC5897) fermented milk (PFM) feeding in newborn mice alleviates allergic symptoms by shifting Th2 to Th1 pathway by decreasing albumin specific antibodies (IgE, IgG, and IgG1), ratio of IgE/IgG2a and IgG1/IgG2a and IL-4, and by increasing IFN-γ, IgA^+ cells, and goblet cells (304, 333).

TABLE 5 | Probiotics therapies in non-infectious diseases.

Probiotics	Disease	Study models	Major finding	References
L. rhamnosus (MTCC5897) fermented milk (PFM)	Allergy	Mice	↑ IgA^+ cells in small intestine ↑ Goblet cells number ↓ Ovalbumin-specific antibodies (IgE, IgG, IgG1) ↓ Ratio of IgE/IgG2a and IgG1/IgG2a ↓ Allergic symptoms	(304)
L. plantarum 06CC2	Allergy	Mice	↓ Ovalbumin-specific IgE ↓ Total IgE ↑ Antiallergic IL-4 and IFN-γ ↓ Allergic symptoms	(305)
Bifidobacterium infantis CGMCC313-2	Allergy	Mice	↓ IL4, IL13 ↓ IgE, IgG1 ↓ Allergic symptoms	(306)
Enterococcus faecalis FK-23	Allergy	Mice	↓ IL-17 ↓ $CD4^+$ cells ↓ TH17 development ↓ Allergic symptoms	(307)
Staphylococcus succinus 14BME20	Allergy	Mice	↓ IgE level in serum ↓ Inflammatory cells flux into lungs ↑ $CD4^+CD25^+Foxp3^+$ regulatory T (Treg) ↑ DCs ↑ IL-10	(308)
Clostridium butyricum CGMCC0313	Allergy	Mice	↓ β-lactoglobulin-mediated intestinal anaphylaxis Inverts the imbalance between Th1/Th2 and Th17/Treg cells ↑ forkhead box P3 (FOXP3) Treg cells ↑ TGF-β and IL10	(309)
L. acidophilus KLDS 1.0738	Allergy	Mice	↓ Inflammatory cells ↓ IgE production ↓ IL6 levels ↓ Th17 response ↑ Treg cells, CD25, FOXP3 ↓ TGF-β	(310)
L. fermentum MTCC: 5898-fermented milk	Cardiovascular	Mice	↓ TNF-α and IL-6 ↓ Coronary artery risk index ↓ Atherogenic index ↓ Triacylglycerols, low-density lipoprotein cholesterol, hepatic lipids ↓ Lipid peroxidation	(311)
L. rhamnosus MTCC: 5957 and *L. rhamnosus* MTCC: 5897	Cardiovascular	Wistar rat	↓ TNF-α and IL-6 ↓ hyperlipidemia ↓ Hepatic lipids ↓ Lipid peroxidation ↑ Antioxidant activities	(312)
L. plantarum	Cardiovascular	Meta-analysis of randomized controlled trials of 653 participants	↓ Diastolic and systolic blood pressure ↓ Total serum cholesterol ↓ Low-density lipoprotein cholesterol levels ↓ Atherosclerosis index ↓ Hepatocyte steatosis risk	(313, 314)
L. fermentum CECT5716 and *Bifidobacterium breve* CECT7263	Cardiovascular	Wistar Kyoto rats	↓ Hypertensions ↓ Endothelial dysfunctioning ↓ Increased blood pressure	(315)
L. rhamnosus GR-1 *L. plantarum* 299v	Cardiovascular	rats	↓ Risk of myocardial infarction Improve ventricular function ↓ Infarct size ↓ levels of leptin	(316, 317)
L. rhamnosus MTCC: 5957, *L. rhamnosus* MTCC: 5897, and *L. fermentum* MTCC: 5898	Diabetes	Wistar rat	Improve glucose metabolism (fasting blood glucose, glycated hemoglobin, serum insulin) Improve serum inflammation status (TNF-α and IL-6) Improve serum lipid profile	(318)

(Continued)

TABLE 5 | Continued

Probiotics	Disease	Study models	Major finding	References
L. plantarum, *L. helveticus*, *L. lactis*, *L. pentosus*, *L. paracasei*, *L. paracasei sbusp.tolerans*, *L. mucosae*, *L. rhamnosus*, *L. harbinensis*, *L. hilgardii*, *Issatchenkia orientalis*, *Candida ethanolica*, *Kluyveromyces marxianus*, and *Pichia membranifaciens*	Diabetes	db/db mice and C57BL/KS	Prevent pancreatic cell apoptosis *via* upregulation of the PI3K/AKT pathway and increase GATA like protein 1 (GLP1) production. GLP1 induces insulin secretion by upregulating the G protein-coupled receptor 43/41 (GPR43/41), proconvertase 1/3 and proglucagon activity	(319)
L. fermentum NCIMB	CNS	Mice	↑ Ferulic acid ↓ Alzheimer's disease symptoms ↓ Oxidative stress and neuroinflammation	(65)
L. reuteri	CNS	Mice	Behavioral improvement	(296)
L. rhamnosus JB-1	CNS	Mice	↓ Gamma aminobutyric acid receptor and corticosterone levels	(300)
L. rhamnosus, *E. faecium*, *L. acidophilus*, *Bifidobacterium bifidum*, and *Bifidobacterium longum*	Obesity	*In vivo* human trial	↓ Low density lipoprotein cholesterol ↓ Total cholesterol ↓ Oxidative stress	(320)
Bifidobacterium bifidum W23, *Bifidobacterium lactis* W51&W52, *L. lactis* W19&W58, *L. brevis* W63, *L. casei* W56, *L. acidophilus* W37, and *L. salivarius* W24	Obesity	*In vivo* human trial	↓ Homocysteine ↓ Triglyceride ↓ Total cholesterol ↓ TNF-α	(321)
L. plantarum CBT LP3, *Bifidobacterium breve* CBT BR3	Obesity	*In vivo* human trial	Reduced obesity marker	(320)
L. fermentum NCIMB 5221	Obesity	–	↑ Ferulic acid ↓ Obesity	(65)
L. fermentum NCIMB	Cancer	AGS, HeLa, MCF-7, and HT-29 cells	↓ Risk of cancer	(65)
L. casei	Cancer	Colonic epithelial cells and HT29 cells	↓ Adenoma atypia ↓ DNA damage	(322)
L. acidophilus	Cancer	Breast cancer mouse model	↓ Tumor growth	(323)

Bifidobacteriales, *Bacteroidales*, and *Lactobacillale*s in the gut affect the activities of inhaled allergens. *Bifidobacteriales* and *Lactobacillales* suppress allergen sensitization and are effective against allergic rhinitis (334). *B. infantis* CGMCC313-2 represses allergen-mediated inflammatory cells, IL4, IL13, IgE, IgG1, and blunt inflammation during allergy in mice model (306). *E. faecalis* FK-23 inhibits the development of Th17 cells in the intestine, spleen, and lungs of infected mice by inhibiting the expression of TGF-β and IL6 mRNA, thereby facilitating to reduce ovalbumin-induced allergic complication (307). *Staphylococcus succinus* 14BME20 has also shown antiallergic potential; it significantly decreases the influx of inflammatory cells into the lungs, suppresses airway hyperresponsiveness, and reduces the serum IgE and Th2 cells cytokines production in an ovalbumin mice model (308). *Clostridium butyricum* CGMCC0313 increases forkhead box P3 (FOXP3) Treg cells, TGF-β, and IL10, inverts the imbalance between Th1/Th2 and Th17/Treg cells, and reduces β-lactoglobulin-mediated intestinal anaphylaxis, thereby contributing to the reduction of the risk of allergy in mice (309). Orally administered *L. acidophilus* KLDS 1.0738 ameliorates allergic symptoms by increasing Treg cells, CD25, FOXP3, and TGF-β mRNA expression, and inhibiting inflammatory cells, IgE production, IL6 levels, and Th17 response in mice (310).

Many probiotics are used for the prevention and treatment of diabetes (**Table 5**). *L. rhamnosus* MTCC: 5957, *L. rhamnosus* MTCC: 5897 and *L. fermentum* MTCC: 5898 feeding Improves glucose metabolism (fasting blood glucose, serum insulin, and glycated hemoglobin), oxidative stress (glutathione peroxidase, superoxide dismutase, catalase activity, and thiobarbituric acid reactive substances,) serum inflammation status (TNF-α and IL-6) and serum lipid profile in diabetic rats, and also significantly reduces mRNA expression of gluconeogenesis related genes (pepck and g6pase) (318). *L. acidophilus* KLDS 1.0901 shows antidiabetic characteristics by reducing glycosylated hemoglobin, fasting blood glucose level, and increasing the level of glucagon-like peptide 1 in the serum of mice. Further, *L. acidophilus* KLDS 1.0901 increases glutathione peroxidase and superoxide

dismutase activities and also increases the level of glutathione with the reduction of malondialdehyde level in mice serum (335). Similarly, *L. paracasei* 1F-20, *L. fermentum* F40-4, *Bifidobacterium animalis* subsp. *lactis* F1-7 also exhibit the potential to manage the diabetic problem as shown by the *in vitro* study of Zhang et al. (241) using CACO-2, STC-, RAW246.7, and HepG2 cells in which these probiotics increase glucagon-like peptide 1 and peptide YY hormones and decrease IL-6 and TNF-α levels (336).

Different species of other *Lactobacilli* and yeast strains act also as antidiabetic, preventing pancreatic cell apoptosis *via* upregulation of the PI3K/AKT pathway and increased GATA-like protein 1 (GLP1) production. GLP1-induced insulin secretion by upregulating the G protein-coupled receptor 43/41 (GPR43/41), proconvertase 1/3, and proglucagon activity in mice (319). GLP1 is an antidiabetic hormone involved in glucose homeostasis, and reduction of glucagon secretion and appetite (337–339). Many probiotics improve glucose metabolism (340) and inhibit NF-κB pathway overactivation. NF-κB is associated with diabetes and its inhibition leads to improvement in insulin sensitivity (94, 309). Probiotics reduce the risk of diabetes by regulating different cellular signaling pathways and the expression of sugar metabolism hormones.

Some probiotics improve sperm maturation; *L. casei* and *B. lactis* enhanced the maturation of sperm in diabetic rats and decreased their glucose levels (341). *L. rhamnosus* increased the mRNA expression of androgen receptors α and β, activin and progesterone receptor 1, serum follicle-stimulating hormone, luteinizing hormone, and testosterone. These effects were associated with improvement in spermatogenesis, sperm motility, and sperm production, along with a decrease in the percentage of immotile sperm (342, 343). *Bacillus amyloliquefaciens* has shown similar beneficial effects on semen density, live sperm, and overall quality in breeder chicken (344).

Probiotics are also widely applied to cardiovascular diseases; they significantly decrease hypertension, oxidative stress, blood pressure, inflammatory mediators, and cholesterol levels (311, 345–348). It is observed that cholesterol-enriched fed mice show significantly higher levels of serum triacylglycerols, total cholesterol, low-density lipoprotein cholesterol, atherogenic index, lipid peroxidation, coronary artery risk index, and IL-6 and TNF-α in the liver whereas significantly lower levels of catalase, anti-oxidative enzymes activities, glutathione peroxidase, and superoxide dismutase in the kidney and liver. Whereas, *L. fermentum* MTCC: 5898-fermented milk improves these adverse physiological conditions (311). Similarly, feeding of *L. rhamnosus* MTCC: 5957 and *L. rhamnosus* MTCC: 5897 maintains healthy liver and kidney conditions of Wistar rats by increasing antioxidant activities and by decreasing lipid peroxidation, diet-induced hypercholesterolemia in the feces, kidney, liver, and blood of the rats. These probiotics also reduce the expression of mRNA of the TNF α and IL-6 inflammatory markers (312).

Lactobacillus plantarum has shown beneficial effects during the meta-analysis of a randomized controlled trial of 653 participants having cardiovascular diseases, lower diastolic and systolic blood pressure (313), total serum cholesterol, low-density lipoprotein cholesterol levels, atherosclerosis index, and hepatocyte steatosis risk. Furthermore, *L. plantarum* decreases liver triglyceride and cholesterol, whereas it increases cholesterol in feces and excretion of bile acid (314). *In vivo* study of Robles-Vera et al. (315) showed that *L. fermentum* CECT5716 and *Bifidobacterium breve* CECT7263 feeding prevent the development of hypertension, endothelial dysfunctioning, and increase in blood pressure in rats (315). *L. rhamnosus* GR-1 and *L. plantarum* 299v reduce the risk of myocardial infarction, improve ventricular function, and reduce the infarct size by decreasing the levels of leptin in rats (316, 317). Probiotics also decreased the levels of toxic circulating metabolites (indoxyl-sulfate and p-cresyl sulfate) associated with cardiovascular diseases and reduced mortality in patients undergoing dialysis (349). Probiotics exert beneficial effects on cardiovascular diseases through different mechanisms of action (i.e., improving the ratio of low-density and high-density lipids, lowering cholesterol levels, improving endothelial function, and regulating the immune cells) (**Table 5**).

CONCLUSIONS AND FUTURE PROSPECTS

Due to increasing antibiotic-resistant bacteria and antibiotic side effects, the use of antibiotics as a feed supplementation is prohibited in many countries. China also bans the supplementation of growth-promoting antibiotics in animal feed since January 1, 2021. Probiotics are considered as a good alternative for antibiotics, providing an alternative treatment option. Probiotics are widely used in human aquaculture, livestock, and poultry to promote health and counteract enteric pathogens. Probiotics are widely used for the management and treatment of bacterial, viral, parasitic infections as well as non-infectious disorders like mental disorders, cancer, allergies, and metabolic disorders. Concerning their mechanisms of action, probiotics have immunomodulatory and many other mechanisms of action, and work in diverse ways to exert beneficial effects on their hosts, if applied properly. However, concerning the safety and efficacy of probiotics, recent screening techniques rely on the capacity of microbes to elicit cytokine production mostly through cell lines or *ex vivo* isolated residual immune cells, even though they do not reflect the phenotype of intestinal cells. Awareness of the capability and usage of probiotics to improve the microbiota equilibrium in the host gut, to serve as immunomodulators, growth promoters, and to inhibit pathogenic infections is crucial from a practical point of view. It will help to make more progress by investigating more expertise, knowledge, and research on the understanding of probiotics, their specific mechanism of action, and their complete applicability for the safety of the host. More importantly, the safety of probiotics during application should also be carefully considered and strictly evaluated in the future in case of the emergence and spread of antibiotic-resistant bacteria between hosts. Thus, high-throughput validation approaches, as well as comprehensive and credible clinical, *in vivo*, and *in vitro* research on probiotic administration are

warranted to clearly illustrate the advantages and adverse effects of probiotics.

AUTHOR CONTRIBUTIONS

SC and LL designed, modified, and reviewed the manuscript. AR and GZ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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ILC3, a Central Innate Immune Component of the Gut-Brain Axis in Multiple Sclerosis

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Gut immune cells have been increasingly appreciated as important players in the central nervous system (CNS) autoimmunity in animal models of multiple sclerosis (MS). Among the gut immune cells, innate lymphoid cell type 3 (ILC3) is of special interest in MS research, as they represent the innate cell counterpart of the major pathogenic cell population in MS, *i.e.* T helper (Th)17 cells. Importantly, these cells have been shown to stimulate regulatory T cells (Treg) and to counteract pathogenic Th17 cells in animal models of autoimmune diseases. Besides, they are also well known for their ability to stabilize the intestinal barrier and to shape the immune response to the gut microbiota. Thus, proper maintenance of the intestinal barrier and the establishment of the regulatory milieu in the gut performed by ILC3 may prevent activation of CNS antigen-specific Th17 cells by the molecular mimicry. Recent findings on the role of ILC3 in the gut-CNS axis and their relevance for MS pathogenesis will be discussed in this paper. Possibilities of ILC3 functional modulation for the benefit of MS patients will be addressed, as well.

Keywords: ILC3 cells, multiple sclerosis, gut-associated lymphoid tissues (GALT), Treg - regulatory T cell, Th17 (T helper 17 cell), AhR (Aryl hydrocarbon Receptor), FFAR2 (GPR43), TLR2

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INTRODUCTION

One of the major open questions about multiple sclerosis (MS) pathogenesis is how the autoimmune response directed against the central nervous system (CNS) is initiated. It is not only that we have not been able to identify preliminary antigens that the autoimmunity is directed against, but also the place of the initial activation of the autoimmune response remains elusive. Gut microbiota has been increasingly studied as the source of antigens that can activate CNS-specific autoreactive T cells, while gut-associated lymphoid tissues (GALT) have been considered as the potential site of their initial activation. MS pathogenesis essentials are presented in **Box 1**, while details can be found in numerous review papers (1–4). In the following chapters, we will present current knowledge on the role of gut microbiota and GALT in the etiopathogenesis of MS, with an emphasis on the role of intestinal innate lymphoid cells type 3 (ILC3) in the process.

BOX 1 | Multiple sclerosis (MS) essentials

- MS is chronic inflammatory, demyelinating and neurodegenerative disease of the central nervous system (CNS).Typical neurological symptoms of MS comprise diminished sensory and visual perception, motor dysfunction, fatigue, pain, and occasionally cognitive deficit. Most MS patients exhibit a relapsing-remitting course of the disease, distinguished by alternations between acute attacks and remission phases. Also, MS may present clinically isolated syndrome or progressive (primary and secondary) clinical course.
- Autoimmune response against the CNS resulting in the CNS inflammatory infiltrates has a major contribution to MS pathogenesis.IFN-γ-producing Th1 cells and IL-17-producing Th17 cells, defined by the expression of T-bet and RORγt master regulators, respectively, enter the brain at semipermeable and damaged sites of the BBB and initiate neuroinflammation. Neuroinflammation induces the opening of BBB and enables the second wave of immune cell entry into the CNS and the formation of brain lesions. $CD8^+$ T cells, B cells, and macrophages (Mf) have the leading role in the CNS tissue destruction $CD4^+$ T regulatory cells (Treg), defined by the expression of CD25 and Foxp3 as a master transcription factor, operate at the opposite arm of neuroinflammation to reduce/recover damage.
- The etiology of MS is multifactorial and involves interaction between intrinsic (genetic) and extrinsic (environmental) risk factors that influence either innate or adaptive immunity. Despite the conclusive autoimmune trait of MS, the precise trigger for the CNS-directed autoimmune response is still unknown.Autoreactive T cells in the blood of MS patients display specificity for multiple myelin protein-derived antigens such as myelin basic protein (MBP), proteolipid protein, and myelin oligodendrocyte glycoprotein (MOG). However, none of these myelin protein-derived antigens is recognized as a dominant antigen in MS, while T cells of the same specificity exist in the blood of healthy individuals. Experimental autoimmune encephalomyelitis (EAE) is an animal model of MS that is induced in susceptible animals through immunization with the CNS antigens.

THE GUT IMMUNE SYSTEM IN THE INTRICACY OF THE CNS AUTOIMMUNITY

Apart from the induction of immune responses against harmful microorganisms and maintaining immune homeostasis in the gut, the immune system of the gut intercedes between intestinal microbiota/metabolites and autoimmune responses. Immune cells are highly enriched in the GALT organized in the forms of Peyer's patches, isolated lymphoid follicles, and scattered among the intestinal epithelial cells and in the lamina propria across the gastrointestinal tract. Also, the immune system of the gut encompasses gut-draining lymph nodes that have intensive communication with the GALT. Recently disclosed changes in immune cells composition and accumulation within different GALT compartments in EAE animals (5–13) support the concept that initiation and/or regulation of autoimmune response to CNS antigens may occur in the gut.

The gut microenvironment participates in the shaping of autoimmune responses to CNS antigens presumably by modulating the activation/differentiation of autoreactive T cells and guiding their trafficking to the CNS. Potentially encephalitogenic T cells were shown to migrate into the gut, where they were further activated towards pathogenic population, or they were modulated to become regulatory cells (7, 14, 15). Accordingly, enhanced Th17 induction in response to segmented filamentous bacteria was described in the small intestine of mice, in particular in the terminal ileum (16–18), while excessive Th17 expansion in the small intestine of humans was associated with MS activity (19). Also increased numbers of Th1/Th17 cells and decreased numbers of Treg cells were found in the gut lamina propria, Peyer's patches, and mesenteric lymph nodes of mice with experimental autoimmune encephalomyelitis (EAE) before the appearance of clinical symptoms, as well as at the disease peak (9). Increased intestinal permeability, alterations in tight junction functioning, and modifications in intestinal morphology occurred along with the changes in the T cells composition in GALT, thus indicating that disruption of intestinal homeostasis was dependent on the immune response at the initiation of EAE (9). Even more, it has been suggested that the very initiation of MS may occur in the GALT through the process of molecular mimicry and/or as a consequence of the loss of gut barrier integrity (20–22).

Conversely, GALT is involved in establishing tolerance to orally administered (auto)antigens including peptides from the nervous tissue. Increased apoptosis of autoreactive T cells in myelin basic protein (MBP)-fed mice occurs in Peyer's patches, thus indicating that Peyer's patches are the principal site for oral tolerance induction in the MBP-specific model of EAE (5). Furthermore, suppression of EAE induced by CD3-specific antibody treatment was presumably reflected by conversion of myelin oligodendrocyte glycoprotein (MOG)-specific Th17 cells into regulatory phenotype occurring in the small intestine (7). It is assumed that autoreactive T cells experiencing phenotypic adaptation in the GALT attain characteristics that favor their migration to the brain (20). Trafficking of CNS-specific autoreactive cells to the gut is mediated through α4β7-MAdCAM-1 (mucosal addressin cell adhesion molecule 1) interaction. Protection from MOG_{35-55}-induced EAE in MAdCAM-1-deficient mice was accompanied by impaired migration of MOG_{35-55}-activated lymphocytes to small intestine lamina propria and Peyer's patches (12). Infiltration of colonic lamina propria with MOG-specific Th17 cells, also dependent on α4β7-MAdCAM-1 pathway, in the preclinical phase of EAE, has been demonstrated in both active and adoptive transfer EAE models in mice (13). These findings support the notion that recruitment of encephalitogenic T cells to the GALT occurs before immigration into the CNS. However, data are showing that IL-4, co-expressed in Th17 cells or used for treatment in EAE mice, redirected trafficking of pro-inflammatory Th17 cells from the CNS and draining lymph nodes to the mesenteric lymph nodes and ameliorated the disease (10). This effect was achieved through IL-4 dependent increase of retinoic acid (RA) production in dendritic cells (DC) and further induced expression of gut-homing receptors CCR9 and α4β7 on Th cells. Moreover, retaining the autoreactive pro-inflammatory T cells within the intestine has

been associated with the resistance to EAE induction in mice (15). It seems that GALT controls CNS-directed autoimmune responses by providing a microenvironment for the activation and differentiation of both encephalitogenic Th cells and Tregs (that may halt these autoreactive T cells). The relationship between the gut and the CNS autoimmunity is shown in **Figure 1**.

Different subpopulations of immune cells residing in GALT that might contribute to CNS autoimmunity comprise conventional lymphocytes ($CD4^+$ Th cells, Tregs, $CD8^+$ T cytotoxic cells), antigen-presenting, and phagocytic cells (DC and macrophages - Mf), and non-conventional lymphocytes, i.e., ILC. Recent findings disclosed the crucial role of the TGF-β-Smad7 regulatory pathway in the generation of CNS autoreactive Th cells in the intestine as Smad7 inhibited induction of Treg by TGF-β (23). Furthermore, decreased TGF-β signaling with a shift toward inflammatory T cell subtypes was demonstrated in intestinal biopsies from MS patients (23). However, it is acknowledged that TGF-β in combination with pro-inflammatory cytokines promotes Th17 differentiation. Intestinal DC expressing αvβ8 were shown to convert latent TGF-β to an active form and thus favor the generation of Th17 and IL-17-mediated CNS inflammation (24, 25). Besides, in EAE mice the frequency of DC was inversely correlated with the frequency of $CD39^+$ Tregs in GALT (26). Considering that DC in GALT present primarily the target for manipulation of orally induced tolerance, it was shown that in orally-tolerated EAE mice intestinal lamina propria γδ T cells secrete XCL1 to promote migration of tolerogenic DC to mesenteric lymph nodes where they induce Tregs (27). Gut-derived IgA-secreting plasma cells in the CNS were recently shown to limit neuroinflammation *via* the production of IL-10 (28). Conversely accumulation of IgA-producing cells reactive with gut bacterial strains associated with MS correlated with acute inflammation in MS (29).

The recently identified ILC primarily involved in regulating intestinal immune responses have also been implicated in CNS autoimmunity. Among different subpopulations of ILC, ILC3 have raised special attention due to the functional similarities with the Th17 that are the major players in CNS inflammation. Indeed, ILC3 share the signature transcription factor retinoid-related orphan receptor γt (RORγt) with Th17 and produce the same major cytokines as Th17 (30).

ILC3 AS THE CENTRAL REGULATORS OF THE GUT IMMUNITY

Immature ILC develop in the bone marrow from common lymphoid progenitor and they tend to migrate to mucosal tissues, although some populate lymphoid tissues, including the spleen and lymph nodes and non-lymphoid organs, such as liver, brain and pancreas (31–34). Also, differentiated ILC3 were found in the bloodstream during a T-cell mediated autoimmune inflammatory disease such as psoriasis (35). ILC3 diverge into at least two subsets that differ developmentally, phenotypically and functionally. Lymphoid tissue inducer cells (LTi)-like ILC3 are characterized by surface expression of CCR6, while natural cytotoxicity receptor $(NCR)^+$ ILC3 express NKp46 in mice (36).

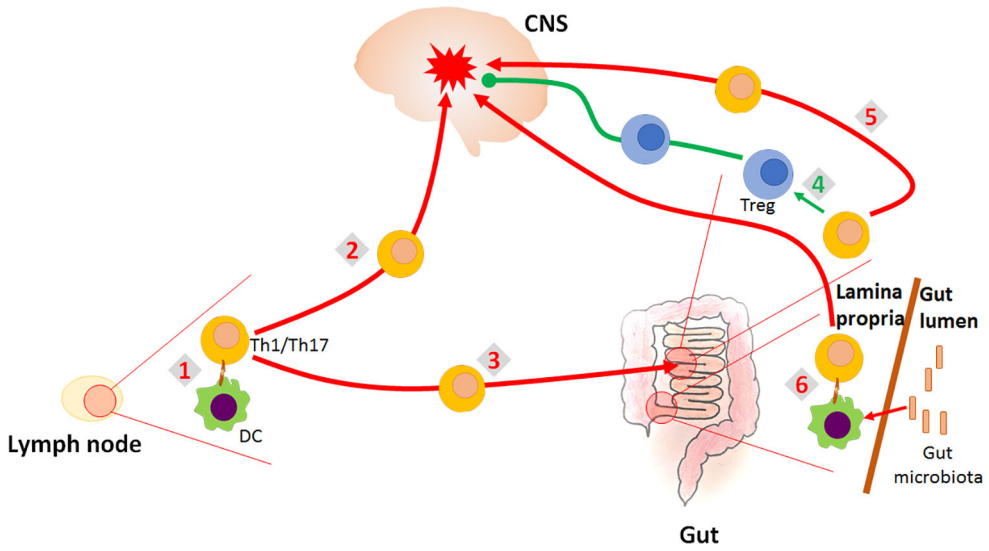

FIGURE 1 | Role of the gut in the CNS autoimmunity. CNS-autoreactive Th1 and Th17 cells are activated in the lymph nodes (1). They migrate into the CNS where they initiate inflammatory response imposing destruction of the CNS tissue (2). They also migrate into the gut (3), where they can be re-differentiated to Treg which counteract the inflammation in the CNS (4). However, they can also be supported by the gut environment in their encephalitogenicity (5). Finally, it is proposed that encephalitogenic Th cells might be initially activated in the gut by the process of molecular mimicry, as they cross-react with gut microbial antigens (6).

Mature ILC3 develop in the lamina propria of the intestine due to specific differentiation factors (retinoic acid - RA, polyphenols and microbiota) (37). Once ILC3 populate tissues, they usually do not migrate (38), thus they have to be replenished through regular divisions. Gut ILC3 proliferation is stimulated by cytokines, including IL-18, tumor necrosis factor-like cytokine 1A, IL-1β, IL-23 and IL-2 (39, 40), short-chain free fatty acids (SCFA) and vitamins A and D (41, 42). ILC3 are critical for the generation of the organized lymphoid tissue in the intestinal wall during development, and they regulate microbiota content and the integrity of the intestinal barrier (38, 43).

ILC3 are present in different GALT compartments where they closely interact with other immune cells, including Th1 and Th17 cells, as well as with the major regulatory population of T cells – FoxP3$^+$ T cells, i.e. Treg (14). It is assumed that the healthy balance between Th17 and Treg in the gut is the major prerequisite for adequate functioning of the adaptive immune system and prevention of autoimmune diseases. The ratio and function of Treg and Th17 in the gut are largely under the influence of gut microbiota and food constituents (44). It has been documented that ILC3 can efficiently control the effector Th1 and Th17 cells and shift T effector/Treg balance to the regulatory side (45–47).

ILC3 can sense cues originating from the food or microbiota as they express numerous receptors, such as retinoic acid receptor (RAR) (48), vitamin D receptor (VDR) (49), aryl hydrocarbon receptor (AhR) (43, 50), and free fatty acid receptors (FFAR) (51). In response to environmental signals, such as vitamins, indoles, SCFA, as well as to cytokines produced by surrounding cells, ILC3 produce several cytokines, including IL-17A/F, IL-22, GM-CSF and IL-2.

The main role of IL-17 produced by ILC3 is to attract neutrophils to the intestinal tissue in response to bacterial and fungal infections (52–54). ILC3-derived IL-17 is also important for the induction of antimicrobial peptides and tight junction proteins (55).

ILC3 react to IL-1β produced by gut microbiota-stimulated antigen-presenting cells (DC/Mf) by secreting IL-2 which potentiates Treg activity (47), and GM-CSF which stimulates the release of IL-10 and RA from DC/Mf (56). IL-10 and RA also stimulate Treg activity. Of specific interest for the homeostasis in the gut are IL-2-producing ILC3 (47), as they are essential for IL-2-mediated Treg cell maintenance and, consequently, for oral tolerance to dietary antigen in the small intestine. Further, OX40L-expressing ILC3 were shown extremely important for Treg homeostasis in the intestine (57). Also, ILC3 drive the differentiation of T cells towards Treg as they present antigens within MHC class II molecules to T cells, but without co-stimulatory signals (45). Further, gut ILC3 present antigens to effector Th17/Th1 cells, yet without adequate co-stimulation (58), thus causing their inactivation. Even with OX40L expression, MHC class II$^+$ ILC3 were shown to regulate effector T cells in acute colitis (59). Thus, ILC3 act in two ways: directly on effector Th17/Th1 cells or through potentiation of Treg that suppress the effector cell activity.

ILC3 are an important source of IL-22, the key cytokine for the stabilization of the intestinal barrier (57). IL-22 keeps intestinal barrier integrity through stimulation of gut epithelial cell turnover, induction of tight junction proteins production, as well as by stimulation of anti-bacterial peptides and mucins generation (60–63). IL-22 and lymphotoxin α produced by ILC3 have the dominant role in epithelial fucosylation involved in the formation of an environmental niche for small intestine commensal bacteria (64). Production of IL-22 by ILC3 is stimulated by multiple biomolecules. IL-1β, IL-18 and IL-23 secreted by DC/Mf stimulate IL-22 production in ILC3 (39, 65–68). ILC3 can recognize lipid antigens through CD1d and consequently generate IL-22 (69). IL-22 production in ILC3 was also shown to be stimulated by a glial-derived neurotrophic factor produced in enteric glial cells in response to TLR ligands (70). Vitamins A and D are potent inducers of IL-22 production by ILC3 (48, 49), as well as AhR ligands and SCFA that act through AhR and FFAR, respectively (50, 71, 72). **Figure 2** illustrates the immunoregulatory activity of gut ILC3 related to CNS autoimmunity.

It has recently been convincingly demonstrated that ILC3 are responsive to circadian regulation (73–75). Importantly, the diurnal rhythm was found affected in EAE (76), while the loss of molecular clock in myeloid cells was found associated with exacerbation of EAE (77). Also, it was reported that IL-22 production in ILC3 and consequent regulation of intestinal barrier function were under the control of vasoactive intestinal peptide (VIP) released from the local enteric neurons (78, 79). VIP release is induced by food consumption, while the functionality of the barrier was inversely correlated with increased growth of epithelial-associated segmented filamentous bacteria. Thus, it is tempting to speculate that disbalanced regulation of the molecular clock in ILC3 contributes to EAE pathogenesis.

Still, it has to be noted that several studies imply pro-inflammatory and disease-promoting activity of ILC3. For example, GM-CSF production by ILC3 was associated with enhanced maturation and polarization of inflammatory intestinal Mf and with the intestinal inflammatory response as observed in colitis (80, 81). Also, MHC class II$^+$ ILC3 were shown to co-stimulate effector T cells in chronic colitis (59). The high salt diet was shown to potentiate IL-17 production in ILC3 and subsequent intestinal inflammation (82). Further, as a part of the gut immune response to segmented filamentous bacteria, ILC3 stimulated epithelial serum amyloid A protein production, which in turn promoted Th17 cells (83).

UNTANGLING POTENCY OF GUT ILC3 MODULATION FOR MS THERAPY

As previously emphasized, ILC3 have a central role in controlling the interaction between the gut microbiota and the host immune system. MS patients were shown to have altered gut microbiota composition, and the alterations were associated with MS pathogenesis [reviewed in (84)]. Some studies directly showed

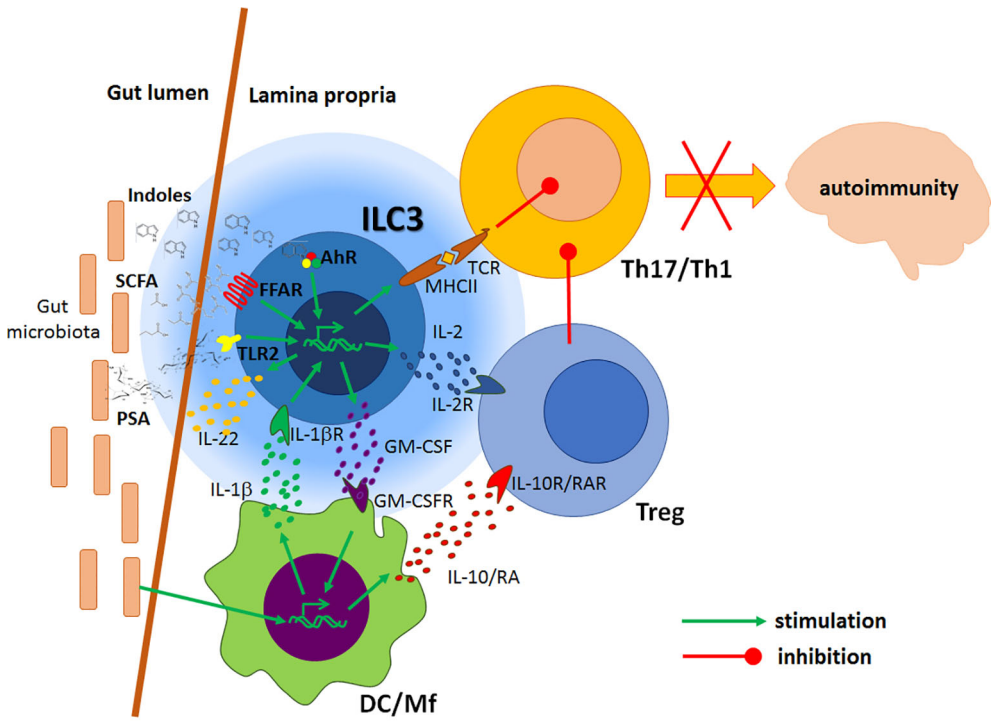

FIGURE 2 | Regulatory effects of gut ILC3 on the CNS autoimmunity. Local antigen-presenting cells (DC/Mf) produce IL-1β under the influence of gut microbiota. IL-1β stimulates ILC3 to produce GM-CSF, which potentiates tolerogenic properties of DC, IL-22 that stimulates intestinal barrier, and IL-2 that favours Treg. DC/Mf also produce IL-10 and retinoic acid (RA) which stimulate Treg activity. ILC3 present antigens to effector Th1 and Th17 cells, but without adequate co-stimulation, thus inhibiting their functions. Products of gut microbiota, such as polysaccharide A (PSA) and processed nutrients, such as short-chain free fatty acids (SCFA) and indoles act on ILC3 through their respective receptors to potentiate their immunomodulatory actions. Consequently, encephalitogenic Th cells are inhibited in the gut, thus ILC3 activity presumably leads to the amelioration of the CNS autoimmunity.

the influence of MS gut microbiota on CNS autoimmunity. In a groundbreaking study performed by Wekerle's group, RR mice that develop spontaneous EAE were transferred with fecal samples obtained from monozygotic twin pairs discordant for MS (85). Germ-free RR mice did not develop EAE, but the disease was initiated through their colonization with human gut microbiota. Importantly, the markedly higher proportion of mice developed EAE in response to MS twin-derived fecal samples than to healthy twin-derived ones. Similar results were obtained in another study, where the transfer of gut microbiota from MS patients to germ-free C57BL/6 mice increased their susceptibility for the induction of active EAE to a greater extent than the transfer of gut microbiota from healthy subjects (86). These studies imply that the dysbiotic gut microbiota of MS patients can be associated with the disease pathogenesis. Indeed, reduced diversity of gut microbiota in MS patients correlated with increased abundance of $CXCR3^+$ T cells expressing the gut-homing α4β7 integrin receptor in the peripheral blood (87). Even more, MS gut microbiota might contain microorganisms that are able to provoke or promote CNS autoimmunity. It was reported that elevated levels of *Akkermansia muciniphila*-specific IgG were present in the cerebrospinal fluid of MS patients (88). Moreover, a $CD4^+$ T cell clone that was clonally expanded in MS brain lesions was shown to recognize guanosine diphosphate-l-fucose synthase, an enzyme expressed by gut microorganisms (21). Accordingly, a recent EAE study has identified specific gut microorganisms that are involved in the reactivation of MOG-specific T cells (22). Namely, peptides originating from *Lactobacillus reuteri* mimic MOG, while *Erysipelotrichaceae* has been shown to act as an adjuvant to enhance the responses of encephalitogenic Th17 cells. Also, gut microbiota composition was shown to change during EAE and to vary between the disease stages and between different clinical subtypes of the disease (89–91). The contribution of gut dysbiosis to the CNS autoimmunity is shown in **Figure 3**, while the possibility to alter gut microbiota for the benefit of MS patients is discussed in **Box 2**.

Thus, it is tempting to speculate that gut dysbiosis observed in MS affects ILC3, as these cells are among the central knots of the gut-CNS MS-related network. Accordingly, it seems reasonable to potentiate the regulatory properties of intestinal ILC3 through modulation of gut microbiota for the benefit of MS patients. Modulation of gut microbiota by antibiotics, pro/prebiotics or fecal microbiota transfer (FMT) is one of the ways to influence ILC3, among the other gut immune cells that are responsive to the changes in the gut microbiota composition and function.

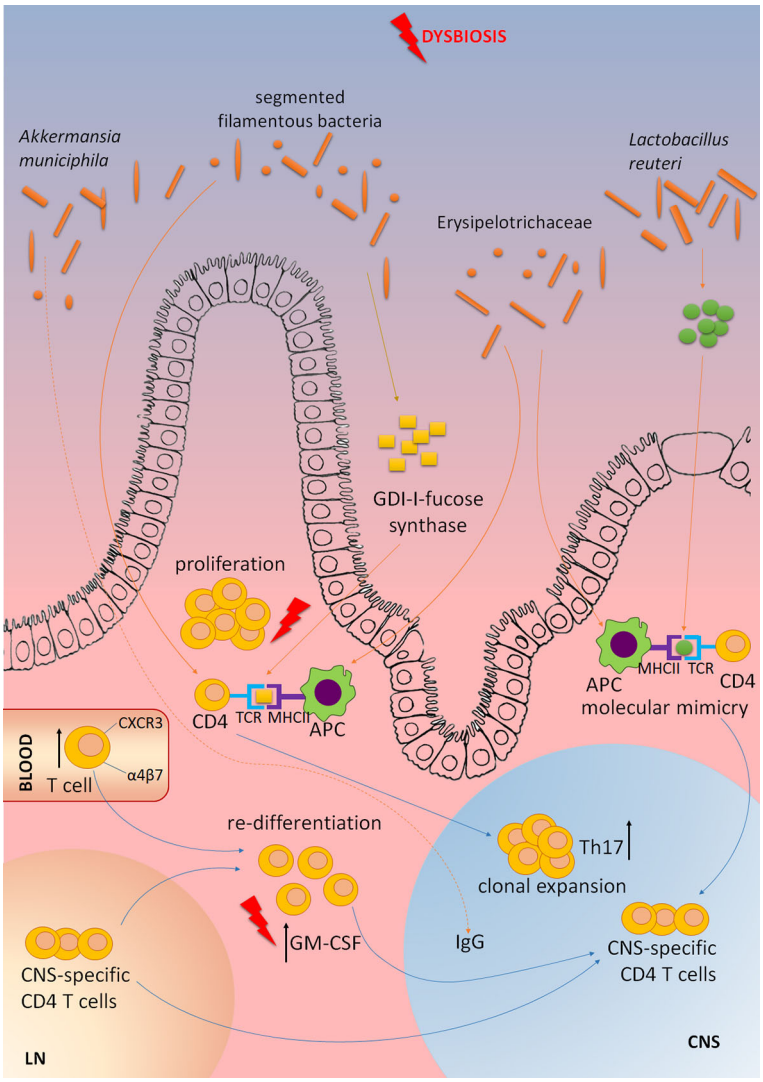

FIGURE 3 | The contribution of gut microbiota dysbiosis to MS pathogenesis. Elevated levels of *Akkermansia muciniphila*-specific IgG are present in the cerebrospinal fluid of MS patients. $CD4^+$ T cell clone that is clonally expanded in MS brain lesions is shown to recognize GDP-l-fucose synthase, an enzyme expressed by gut microorganisms. Peptides originating from *Lactobacillus reuteri* mimic myelin oligodendrocyte glycoprotein (MOG), while *Erysipelotrichaceae* can act as an adjuvant to enhance activity of antigen-presenting cells (APC), and subsequent activation of encephalitogenic Th17 cells. Segmented filamentous bacteria stimulate CNS autoimmunity by inducing Th17 cell differentiation. Dysbiosis of gut microbiota in MS patients correlates with increased abundance of $CXCR3^+$ T cells expressing the gut-homing α4β7 integrin receptor in the peripheral blood. Gut dysbiosis might increase the abundance of GM-CSF-producing $CD4^+$ T cells that are among the major culprits in CNS autoimmunity. CNS-specific T cells originating in lymph nodes (LN) migrate to the gut where they can undergo re-differentiation into potent encephalitogenic cells under the influence of gut microbiota dysbiosis.

Gut ILC3 gene expression profile was shown rather resistant to broad-spectrum antibiotics, unlike ILC1 and ILC2 which had profound changes in the transcriptome (111). Moreover, ILC1 and ILC2 transcriptional profiles were more similar to ILC3 transcriptional profile, under the influence of antibiotics. It will be important to determine if minocycline or some other antibiotic of choice for the treatment of MS, influences regulatory gut ILC3 properties in EAE or other models of MS. Also, dietary fibers could be investigated in conjunction with ILC3 regulatory activity in MS. Yet, it is even more appealing to administer SCFA or agonists of their receptors to potentiate ILC3-mediated CNS autoimmunity amelioration, as discussed in detail below. The effect of FMT on gut ILC3 has not been investigated in MS animal models, and it surely deserves attention.

Specific targeting of gut ILC3 for the benefit of MS patients can be attempted through the application of compounds that influence ILC3 directly or indirectly. Among various compounds that can be used to target gut ILC3, polysaccharide A, AhR agonists, and SCFA are discussed here. Capsular polysaccharide A produced by *Bacteroides fragilis* was extensively studied in the context of CNS autoimmunity. The studies revealed that

BOX 2 | Gut microbiota alteration for MS therapy

Modulation of the gut microbiota that was shown effective in EAE, and investigated in MS trials can be achieved by the application of antibiotics, probiotics, and gut microbiota transfer. Gut microbiota composition modulation by broad-spectrum antibiotics before EAE induction reduced the clinical severity of the disease (92–94), while the therapeutic application was inefficient (95). Still, EAE aggravation as the consequence of broad antibiotic application was observed in rats (89). Minocycline has been considered as a potential therapeutic for MS (96), and its effectiveness in the prevention of clinically isolated syndrome transition into definitive MS was evaluated in a clinical study (97).

Various probiotics were shown safe and efficient in the prophylactic or therapeutic treatment of EAE (6, 98–101). Effects of probiotics were associated with reduced Th1/Th17 presence and activity in lymph nodes draining the site of immunization, in the spleen, and in the blood (100, 101). Probiotics are widely used in humans and are generally safe for prolonged use. However, their ability to modulate the composition of already established gut microbiota or even to re-establish well-balanced gut microbiota after antibiotic-induced depletion is uncertain (102, 103). Maybe the ingestion of prebiotics, i.e. dietary fibers, that help homeostatic bacteria to overwhelm pro-inflammatory ones is a better approach for the treatment of MS. Indeed, there is an ongoing clinical trial: "Prebiotic vs Probiotic in Multiple Sclerosis" (NCT04038541) that is exploring this possibility. Dietary fibers are metabolized by gut bacteria to short-chain fatty acids (SCFA) that were shown to support gut ILC (51).

The efficiency of fecal microbiota transfer (FMT) has been demonstrated in EAE (104, 105). Some preliminary studies of FMT in a limited number of subjects suggest that this approach can be beneficial in MS (106, 107). Although the results of the studies are encouraging, additional data obtained from large cohorts of patients are needed to get insight into the safety and efficiency of FMT for the treatment of MS. Currently, there are two ongoing clinical trials on the application of FMT in MS ("Fecal Microbiota Transplantation (FMT) of FMP30 in Relapsing-Remitting Multiple Sclerosis (MS-BIOME)", NCT03594487; "Safety and Efficacy of Fecal Microbiota Transplantation", NCT04014413).

Numerous data obtained in EAE imply that gut microbiota modulation by antibiotics, probiotics, and by gut microbiota transfer is the feasible way for the prevention and treatment of CNS autoimmunity (108). Still, it has been postulated that appropriate gut immune system development is established under the influence of gut microbiota in the process of "weaning reaction" during the short window of opportunity period, i.e. days 14 to 28 postpartum in mice (109). This reaction is presumably essential for the development of Treg in the gut and prevention of the future inflammatory pathologies in adult organisms. Also, it has been shown that adult gut microbiota composition changes induced by antibiotics and probiotics are not long-lasting, as the gut microbiota tends to get back in the status of the equilibrium with the host genetics (102, 103, 110). Thus, it is reasonable to question if the gut microbiota-directed intervention in adults will be effective in counteracting gut-related inflammatory and autoimmune disorders.

polysaccharide A acted through TLR2 to stimulate Treg, either directly or by the potentiation of tolerogenic DC functions (112, 113). TLR2 is expressed on gut ILC3 (114) and it will be important to determine if polysaccharide A potentiates regulatory effects of gut ILC3 in EAE.

ILC3 can sense diet-based compounds and changes in the gut microbiota through AhR (115). AhR is highly expressed in ILC3 and is essential for the maintenance of their phenotype under inflammatory conditions (116). For instance, kynurenine produced in gut epithelial cells was shown to increase the abundance of IL-22-producing ILC3 (117). The circulating levels of AhR agonists in general and tryptophan metabolites, in particular, are decreased in sera of MS patients (118). Several research papers indicate the beneficial effects of various AhR ligands in the treatment of EAE (118–120). Notably, EAE enhanced by antibiotics-imposed gut microbiota dysbiosis in mice was ameliorated by AhR ligands indole, indoxyl-3-sulfate, indole-3-propionic acid and indole-3-aldehyde, or the bacterial enzyme tryptophanase (118). Thus, the effects of AhR-based interventions on gut ILC3 functional properties in EAE deserve particular attention.

ILC3 express various SCFA receptors, but the highest expression was shown for free fatty acid receptor 2 (FFAR2 or GPR43), while the expression of FFAR3 (GPR41) was much lower. Also, ILC3 have a higher expression of FFAR2 than other ILC populations (51, 111). SCFA are important for ILC3 homeostasis in the gut, as it was demonstrated that dietary fibers metabolized by gut microbiota to SCFA stimulated ILC3 proliferation in the small intestine *via* upregulating mTOR activity (51, 121). Fecal SCFA levels are decreased in EAE (122), as well as in MS patients (123–125). Accordingly, oral application of dietary fibers or SCFA was shown beneficial in EAE, as they promoted Treg and ameliorated the disease (126, 127). Interestingly, effects of propionate were superior to those of acetate and butyrate (127), and it was supplementation of propionic acid to multiple sclerosis patients that led to Treg/Th17 balance shift towards the regulatory arm and the improvement of the disease course (124). The effect on the disease included reduced annual relapse rate, stabilization of the disability, and decreased brain atrophy after three years of propionic acid intake (124). It has been suggested that acetate and propionate stimulate, while butyrate inhibits innate immune cell activity (128). As FFAR2, in contrast to FFAR3, has a higher affinity for binding acetate and propionate than butyrate (128), it is reasonable to assume that specific activation of FFAR2 is the proper way to stimulate ILC3. Indeed, the deficiency of FFAR2 in ILC3 led to a decrease in their homeostatic proliferation and IL-22 production (41). Further, acetate was shown to promote IL-1β-imposed ILC3 production of IL-22 as a part of its beneficial effects in *Clostridium difficile* infection (71), while butyrate reduced abundance of NKp46$^+$ ILC3 in terminal ileal Peyer's patches, decreased GM-CSF expression in ILC3 and consequently reduced Treg and enhanced antigen-specific T-cell proliferation (129). Moreover, increased fecal butyrate levels correlated with EAE aggravation in antibiotic-treated rats (90). Therefore, it seems rational to insist on the application of the selective FFAR2 agonists, such as the one used in the study of Chun and colleagues. This selective FFAR2 agonist acted preferentially on gut ILC3, increasing their abundance and their IL-22 production (41). Thus, investigation of ILC3-mediated effects of the FFAR2 agonist in EAE is warranted.

Proposed therapeutic interventions for the stimulation of ILC3 immunoregulatory activity are outlined in **Figure 4**.

To conclude, a plethora of data indicates that ILC3 have a central role in gut immune homeostasis, which seems to be essential for the prevention of MS etiopathogenesis. Further, as ILC3 express FFAR2 receptor almost exclusively, they can be easily modulated with respective agonists without affecting other

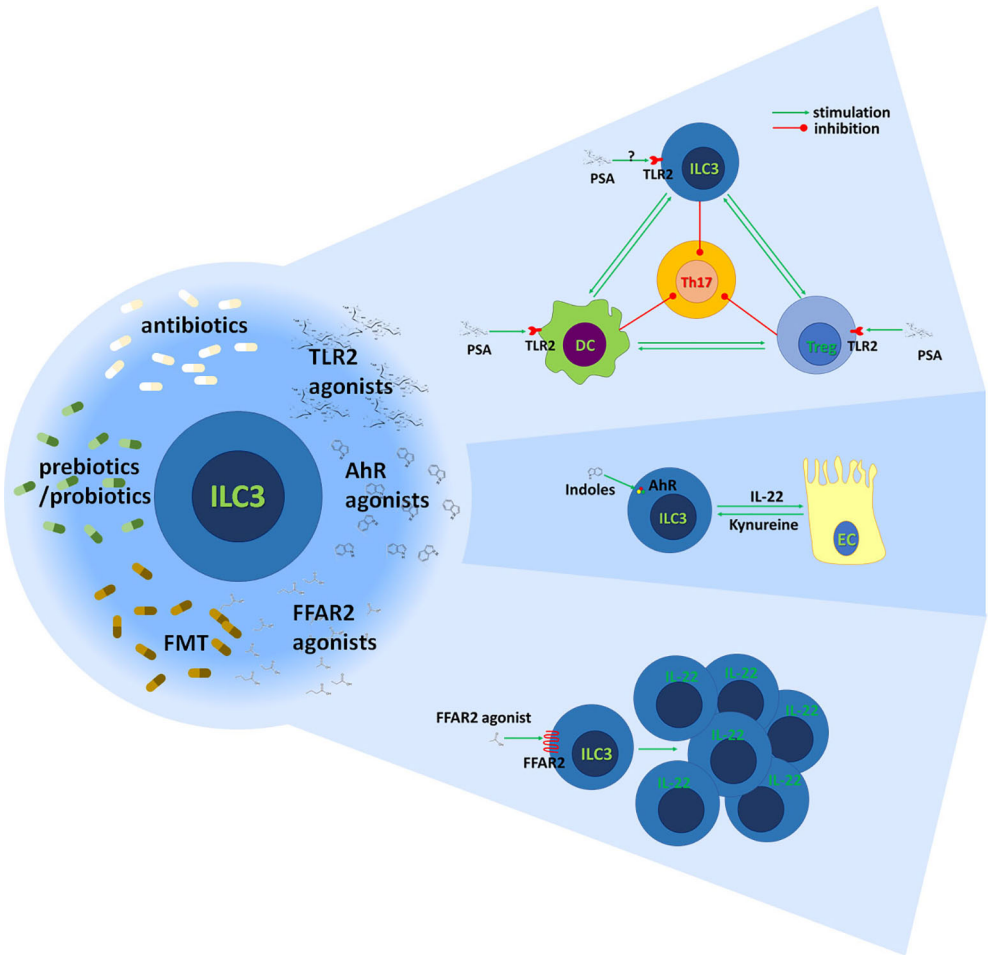

FIGURE 4 | Stimulation of gut ILC3 for the benefit of MS patients. The immunoregulatory activity of gut ILC3 could be achieved through modulation of gut microbiota by antibiotics, prebiotics or probiotics, and fecal microbiota transfer (FMT). Also, it can be potentiated through agonists of TLR2, AhR, and FFAR2. It is known that polysaccharide A (PSA) acts through TLR2 on Treg and DC to inhibit encephalitogenic Th17 cells. Also, it is established that ILC3 potentiate tolerogenic DC properties and stimulate Treg functions. It remains to be determined if PSA acts on ILC3 through TLR2 and if it contributes to ILC3-imposed immunoregulation in the gut. Indole derivates of food and gut microbiota and kynurenine produced by gut epithelial cells (EC) stimulate ILC3 through AhR to generate IL-22. IL-22 has multiple beneficial effects on epithelial cells and the intestinal barrier. FFAR2 agonists promote the proliferation of IL-22-producing ILC3 acting through FFAR2.

immune cells. Thus, the application of FFAR2 agonists is an excellent therapeutic opportunity. A thorough investigation of the role of ILC3 in the pathogenesis of MS, as well as of the possibility to apply ILC3-directed therapy for the benefit of MS patients is a necessity.

AUTHOR CONTRIBUTIONS

All authors drafted the manuscript and participated in the concept design. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.657622/full#supplementary-material

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Aging-Induced Dysbiosis of Gut Microbiota as a Risk Factor for Increased *Listeria monocytogenes* Infection

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Invasive foodborne *Listeria monocytogenes* infection causes gastroenteritis, septicemia, meningitis, and chorioamnionitis, and is associated with high case-fatality rates in the elderly. It is unclear how aging alters gut microbiota, increases risk of listeriosis, and causes dysbiosis post-infection. We used a geriatric murine model of listeriosis as human surrogate of listeriosis for aging individuals to study the effect of aging and *L. monocytogenes* infection. Aging and listeriosis-induced perturbation of gut microbiota and disease severity were compared between young-adult and old mice. Young-adult and old mice were dosed intragastrically with *L. monocytogenes*. Fecal pellets were collected pre- and post-infection for microbiome analysis. Infected old mice had higher *Listeria* colonization in liver, spleen, and feces. Metagenomics analyses of fecal DNA-sequences showed increase in α-diversity as mice aged, and infection reduced its diversity. The relative abundance of major bacterial phylum like, *Bacteroidetes* and *Firmicutes* remained stable over aging or infection, while the *Verrucomicrobia* phylum was significantly reduced only in infected old mice. Old mice showed a marked reduction in *Clostridaiceae* and *Lactobacillaceae* bacteria even before infection when compared to uninfected young-adult mice. *L. monocytogenes* infection increased the abundance of *Porphyromonadaceae* and *Prevotellaceae* in young-adult mice, while members of the *Ruminococcaceae* and *Lachnospiraceae* family were significantly increased in old mice. The abundance of the genera *Blautia* and *Alistipes* were significantly reduced post-infection in young-adult and in old mice as compared to their uninfected counterparts. Butyrate producing, immune-modulating bacterial species, like *Pseudoflavonifractor* and *Faecalibacterium* were significantly increased only in old infected mice, correlating with increased intestinal inflammatory mRNA up-regulation from old mice tissue. Histologic analyses of gastric tissues showed extensive lesions in the *Listeria*-infected old mice, more so in the non-glandular region and fundus than in the pylorus. Commensal species like *Lactobacillus*, *Clostridiales*, and *Akkermansia* were only abundant in infected young-adult mice but their abundance diminished in the infected old mice. Listeriosis in old mice enhances the abundance of butyrate-producing inflammatory members of the *Ruminococcaceae/Lachnospiraceae* bacteria while reducing/eliminating beneficial

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commensals in the gut. Results of this study indicate that, aging may affect the composition of gut microbiota and increase the risk of invasive *L. monocytogenes* infection.

Keywords: *Listeria monocytogenes*, listeriosis, aging, dysbiosis, inflammation, gut microbiota, metagenomics

INTRODUCTION

Listeria monocytogenes is a Gram-positive, aerobic/facultative-anaerobic intracellular bacterium that can infect both humans and animals, including livestock, after ingestion of contaminated food. Human listeriosis caused by *L. monocytogenes* can manifest on a variety of syndromes including gastroenteritis, septicemia, meningitis and chorioamnionitis, and is associated with a high mortality rate (20%-30%). Invasive listeriosis is a more severe form of disease and affects certain high-risk groups of the population such as pregnant women and their fetuses, patients undergoing treatment for cancer, AIDS, organ transplant recipients, infants, and the elderly.

The incidence of listeriosis and the relative risk of infection vary significantly among population subgroups (1). Recently Pohl et al. (2) estimated that in the US, the annual incidence of listeriosis for adults ≥70 years was 1.33 cases per 100,000, while the incidence among the general population was 0.28 cases. In Europe, listeriosis incidence has increased among males ≥ 75 years, and females ≥25 years (2). CDC surveillance data suggest that the number of invasive listeriosis cases increases as people age, and for people ≥50 years of age, that increase is doubled for each 10-year increase in age (3). In general, aging is associated with a higher degree of morbidity in elderly populations due to life-stage-dependent changes in host-immune responses and decreasing ability to fight off systemic infections, like *Listeria* infection. Animal model studies have suggested that age-dependent dysregulation of innate immunity can impair adaptive immune responses and, as a result, altered T-helper effector cells cannot maintain a sustained $CD8^+$ T cell cytokine response required for clearance of foodborne pathogens (4, 5). In addition, recent *L. monocytogenes* animal model studies using intragastric inoculation with Lmo-$InlA^m$ strain suggest that dysregulation of the Th1/Th2 response in aging mice may contribute to higher susceptibility to infection (6, 7).

It has recently become evident that gut microbiota has an important role on the host immune system, metabolism, and even behavior of the host (8, 9). Shifts or imbalances in the composition of gut microbiota has been correlated with many immunological, metabolic, and mental disorders (10). Previous research studies demonstrated that changes associated with aging are recognized by the indigenous microbiota that co-evolved together with its host as a part of the holobiont (11, 12). Moreover, commensal intestinal microbiota (or gut microbiota) confers natural resistance (colonization resistance) against orally acquired bacterial pathogens (13). Intestinal microbiota is believed to directly suppress invading pathogens by producing bacteriocins, by competing for nutrients, and indirectly by modulating host defense pathways. Studies (14–16) have shown that structural changes can happen in the gut that result in dysbiosis: a decrease in the number and diversity of beneficial bacteria (e.g. *Bifidobacteria*) and a corresponding increase in the number and diversity of harmful bacteria (e.g. *Clostridia*, *Enterobacteria*). In general, dysbiosis is any change to the composition of resident commensal communities relative to the community found in disease free state which can be characterized by: loss of microbial diversity, change in composition, including blooms of pathobiont and decrease in commensal or potentially beneficial bacteria (15, 16).

The gut microbiota is a very complex and diverse community of commensal bacteria that intimately interacts with the epithelium and underlying mucosal immune cells in the gastro-intestinal tract (17). A recent study by Becattini et al. reported that commensal microbes like the *Clostridiales* act as first line of defense against *L. monocytogenes* infection in mice (18). So, changes in the populations of commensals may increase the susceptibility of the elderly to foodborne infection. Aging is a recognized risk factor for increased susceptibility to listeriosis in humans and mice (2, 5–7), but little is known about the risk of listeriosis as a function of altered gut microbiota due to aging. In other at-risk populations for listeriosis, such as infants or pregnant women, intestinal dysbiosis has been implicated as a factor associated with susceptibility due to altered microbial profiles with marked reduction in *Clostridiales* members and increase in *Proteobacteria* (18, 19).

Our previous studies using a geriatric listeriosis murine model, suggested increased susceptibility to oral *L. monocytogenes* infection in the old mice due to an imbalance of pro- and anti-inflammatory responses (6, 7). We also observed increased Th1/Th2 responses in the old mice that were aged normally. We hypothesize that susceptibility in mice and humans to *L. monocytogenes* infection might depend on the diversity of gut microbiota that can modulate immune response in the gut. Thus, aging-mediated perturbations/shifts of microbiota would further increase the risk of *L. monocytogenes* infection. In the present study, we used a geriatric murine model of listeriosis as a human surrogate of listeriosis for elderly persons to study aging-induced alteration of commensal microbiota as a risk of invasive *L. monocytogenes* infection. Listeriosis-induced perturbation of gut microbiota and disease severity were also compared between young-adult and old mice and correlated with intestinal pathologies.

MATERIALS AND METHODS

Mice

Four-week old female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and allowed to age in-

house under specific pathogen free (SPF) conditions until use. All mice were categorized as young-adult (2-months) or old (20-months). Aging mice were regularly monitored for senescent changes and only healthy mice were used in the experiments.

Ethical Statements

All experiments were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council. The protocol (protocol approval number: BFQ-11-006) was approved by the Food and Drug Administration, Center for Food Safety and Applied Nutrition-Institutional Animal Care and Use Committee (CFSAN-IACUC). All mice were kept at the MOD-1, CFSAN/FDA, AAALAC (American Association for Accreditation of Laboratory Animal Care) accredited animal facility. Approved standard animal husbandry protocol was followed for the care of mice.

Listeria monocytogenes Infection of Mice

L. monocytogenes was grown on BHI agar plates (BBL, Becton and Dickinson, MD) at 37°C for 18 h. Young-adult (n=5–6) and old mice (n=5–6) were gavaged for two consecutive days (day zero and day-1) with *L. monocytogenes* Lmo-*InlA*^{m} (gift from Dr. Wolf-Dieter Schubert & Dr. Thomas Wollert, HZI, Germany) at a dose of 1×10^{6} CFU in 100 µl of PBS. For each age group, control (young-adult control and old control) mice (n=5–6) were gavaged with 100 µl of PBS. The Lmo-*InlA*^{m} strain is a murinized *L. monocytogenes* strain that is capable of invasion of mouse intestinal tissue and results in systemic infection. The Lmo-*InlA*^{m} strain was modified from the wild-type Lmo-EGD strain by exclusively replacing the gene inlA with inlA$^{S192N-Y369S}$ to produce the mutant strain Lmo-*InlA*^{m} (20).

Mice were fasted for 4-6 hours prior to infection. Metal wire-flooring was used to prevent coprophagia. Mice were monitored daily, for 7 days, for clinical signs of disease. Infected mice that became severely sick were euthanized as per FDA-CFSAN-IACUC guidelines. All mice were also weighed and euthanized on day seven of infection. Spleen and liver tissues were collected for enumeration of *L. monocytogenes* colonization by viable colony counts. Fresh feces from the mice were collected in dry-ice and kept frozen at -80°C until use.

Histopathology, Immunostaining, and Intestinal Damage Scoring

Portions of liver tissues were fixed in Bouin's solution (Ricca Chemical, Arlington, TX), washed with ethanol, embedded in paraffin, cut into 3-5-μm sections, and stained with hematoxylin and eosin (H&E). For gastric tissue analyses and histological evaluation, longitudinal segments, including the antrum and corpus plus proximal duodenum, were fixed in Bouin's fixative solution (Ricca Chemical) for 24 h, washed twice with 70% ethanol and embedded in paraffin, cut into 3- to 5-µm sections, and stained with hematoxylin and eosin. For immunohistochemistry, similar 3-µm gastric sections were stained with polyclonal anti-MPO (Myeloperoxidase) antibody (Novus Biochemicals, Littleton, CO), and tissue-bound peroxidase activity was visualized with DAB (3, 3′-diaminobenzidine). Hematoxylin was used for nuclear counter staining. MPO-positive cells were shown in the tissue with an arrow. All slides were scanned and digitally stored using a Nanozoomer (Hamamatsu, Japan) with NDP-view-2 software (Hamamatsu). Scanned H&E images were used for measuring tissue area or height at suitable magnification with NDP-view-2 software. Gastric inflammation was assessed using a modified scoring system, as previously described (21). Briefly, two sections were collected from each stomach, and each region of the stomach (forestomach or cardia, corpus, and antrum) was assessed individually for three parameters; (1) thickening, (2) infiltration of polymorphonuclear cells and (3) infiltration of MNCs (mononuclear cells). Severity was graded based on the absence (0) or presence (1) of each parameter, with polymorphonuclear infiltration further examined (absence or presence) for focal, diffuse, or abscess involvement. Similarly, MNC infiltration was examined for focal, diffuse, or aggregate involvement in the lamina propria. A total score was calculated by summing the score values for each region of the stomach for one section. Results are reported as total damage scores.

Enumeration of *Listeria monocytogenes* Colonization in Liver and Spleen Tissues

For the measurement of *L. monocytogenes* burden, portions of liver and spleen tissue were homogenized in PBS, and replicate serial 10-fold dilutions were plated onto BHI agar plates and incubated overnight at 37° C. Bacterial counts were determined by viable colony count method.

RNA Extraction and Real-Time RT-PCR

Total RNA was extracted from infected and uninfected tissues using Qiagen RNA extraction kits (Qiagen, Valencia, CA). In each case, RNA was reverse-transcribed to yield cDNA using the RT^{2} First Strand kit (Qiagen). Transcripts were measured by Real-time RT-PCR with a CFX96 Real-Time System (BioRad, Irvine, CA) using RT^{2} SYBR Green qPCR and RT^{2} qPCR primers (IFN-γ: PPM03121A, IL-10: PPM03017B, and IL-17:Add IL-17: PMM03023A, IL-23: PMM03763F) from Qiagen-SABiosciences (Frederick, MD). The levels of RNA for the target sequences were determined by melting curve analysis using the Bio-Rad CFX manager software as previously described (7). Normalized levels of each mRNA were determined using the formula $2^{(Rt\ -\ Et)}$, where Rt is the threshold cycle for the reference gene (GAPDH: PPM02946E, Qiagen) and Et is the threshold cycle for the experimental gene ($\Delta\Delta C_T$ method). Data are expressed as arbitrary units.

Mouse Fecal DNA Extraction

Fecal DNA was extracted from infected and uninfected mouse feces using QIAamp DNA mini kits (Qiagen, Valencia, CA). Extracted DNA samples were kept at – 20°C.

DNA Sequencing

DNA sequencing libraries were prepared with the Nextera XT DNA library preparation kit and Nextera indices (Illumina, San

Diego, CA). Libraries were sequenced on a MiSeq platform using a MiSeq 500 cycle version 2 reagent kit (Illumina).

Metagenomic Analyses

Unassembled metagenomic sequencing reads were analyzed using the CosmosID Metagenomics Cloud Application as previously described (22–25) to achieve multi-kingdom microbiome analyses and quantification of organisms' relative abundance. This is defined as the proportion of unique organism-specific k-mers annotated by each database relative to the total number of unique sequencing reads generated for that sample. Briefly, the application utilizes GenBook®, a series of proprietary databases curated extensively by CosmosID Inc. (CosmosID Inc., Rockville, MD, USA), which is composed of over 150,000 microbial genomes and gene sequences representing over 15,000 bacterial, 5,000 viral, 250 protozoan, and 1,500 fungal species, as well as over 5,500 antibiotic resistance and virulence-associated genes. Metagenomic analyses for microbial composition levels based on changes that occurred in the gut microbiome due to *L. monocytogenes* infection were further analyzed using our in-house k-mer database (k=30) (26) for taxonomical identification of microbes to the species level; the total relative abundance of each organism in each sample was determined.

Statistical Analysis

Statistical models for estimating microbial diversity and microbial community comparison methodology and metrics were performed using the STAMP (Statistical Analysis of Metagenomic Profiles) software package (27). The effect sizes and confidence intervals in microbial composition shifts were calculated between groups (uninfected control *vs Listeria*-infected relative to both young-adult and old mice). Gastric damage scoring and mRNA results are expressed as mean ± SEM or mean ± SD. Data were compared by Student's *t* test (unpaired) or ANOVA, and results were considered significant if p values were less than 0.05. At least two independent experiments were performed.

RESULTS

Fecal Microbiota Population Diversity Differs Between Young-Adult and Old Mice

Sequence analyses of DNA extracted from the samples revealed a wide diversity of bacteria, representing over 250 species, 125 genera and 15 bacterial phyla. **Figure 1A** depicts a Krona (28) visualization of all bacteria detected across all mice tested. The predominant phylum of bacteria were the gram-positive *Firmicutes*, *Bacteroidetes* and *Verrucomicrobia* representing 85%, 10% and 4% of total bacterial diversity, respectively. The phylum *Proteobacteria*, which includes a wide variety of pathogens, and *Actinobacteria*, which includes a wide variety of pathogens as well as symbionts, represented only 0.6% and 1% of total bacterial diversity.

We also evaluated whether gut microbiota differed between young-adult (2-months) and old (20-months) female mice. As depicted in **Figure 1B**, the principal component analysis (PCA) of gut microbiota based on their taxonomic abundance, both uninfected (control) young-adult and old mice clustered separately from *L. monocytogenes* infected young-adult and old mice. **Figure 1C** represents the alpha-diversity that evaluated distinguishable richness in taxa that were detected in fecal samples from both young-adult and old mice before and after *L. monocytogenes* infection. All old mice showed an increased alpha-diversity based on species richness (e.g. CHAO1 index (29) as compared to uninfected young-adult mice, and *L. monocytogenes* infection altered that diversity.

We further analyzed the relative abundance of five major phyla of bacteria, which include *Firmicutes*, *Bacteroidetes*, *Verrucomicrobia*, *Actinobacteria*, and *Proteobacteria*, in mice fecal microbiota before and after infection. As shown in **Figure 2**, the majority of the fecal microbiota (~90%) are represented from the phyla *Bacteroidetes* and *Firmicutes*, with the remaining phyla combined, representing less than 7% of the total bacteria. We did not observe any changes in the combined abundance of *Bacteroidetes* and *Firmicutes* phyla with respect to aging in these mice; however, an interplay between increased abundance of *Firmicutes* followed by reduced abundance of *Bacteroidetes* was observed among mice infected with *L. monocytogenes*. This observation is supported by previous studies (9, 30) which suggested an increased *Firmicutes* to *Bacteroidetes* ratio in old mice as compared to young-adult mice. Interestingly, we saw a significant shift in the abundance of *Verucomicrobia* in both young-adult and old mice after *L. monocytogenes* infection, but their abundance was distinctly more in *Listeria*-infected young-adult mice when compared with old-infected mice.

Listeria monocytogenes Infection Alters Microbiota Population Diversity in Young-Adult and Old Mice

In addition, we evaluated the taxonomic differences of fecal microbiota at the family level and compared the distribution based on high-to-low abundances across two age groups and infection status as shown in **Figure 3**. We selected the twelve most abundant families based on k-mer database metagenomics analyses and determined if particular families of bacteria are associated with aging and/or if *Listeria* infection perturbed abundance. *Bacteroidaceae*, *Sphingobacteriaceae* and *Clostridiales_uncl* bacteria all together comprised nearly 80% of the total fecal bacteria in the gut and was not significantly perturbed due to aging. Interestingly only the *Rikenellacae* family was most significantly ($p<0.05$) over abundant within old mice as compared with young-adult mice before infection; their abundance was significantly ($p<0.05$) reduced when old mice were infected. We do not know the reason for such reduction after infection in the old mice. We also saw marked reduction in the order *Clostridiales*, and the *Clostridiaceae* family as the mice aged. The members of the *Lactobaccillaceae* family bacteria, known for bacteriocins production and their protective antimicrobial roles (31–33), were found in low- abundance in old mice as compared

FIGURE 1 | **(A)** Krona visualization of all bacteria detected across all mice tested. **(B)** Principal component analysis (PCA) of the fecal microbiota from young-adult and old C57BL/6 mice infected with *Listeria monocytogenes* (Lm) after 7-days post infection. The clustering on two PCA plots show control (uninfected) versus *L. monocytogenes* infected mice. Each symbol represents one mouse. Principal component analysis scores are plotted based on the relative abundance of total microbiota. Proportion of variance in each principal coordinate axis is denoted in the corresponding axis label. The uninfected control (brown circle) and infected mice (green circle) show clear separation. **(C)** Alpha diversity comparisons based on CHAO1. *Listeria* infection in old mice significantly lowers microbial richness compared with the uninfected old control mice.

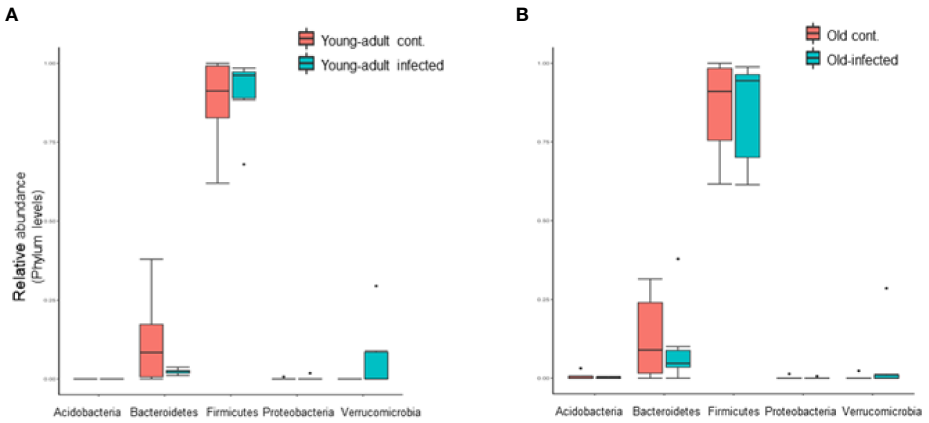

FIGURE 2 | *Listeria monocytogenes* infection induced changes in the relative abundance of fecal microbiota at the phylum level. **(A)** Relative abundance of major bacterial Phylum in young-adult control and *L. monocytogenes*-infected young-adult mice. **(B)** Relative abundance of Phylum bacteria in old control and old *L. monocytogenes* infected mice. Data from mean ± SEM from a representative experiment using 4–6 mice. *p < 0.05.

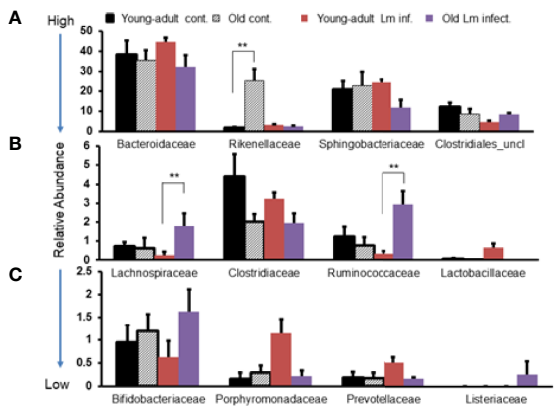

FIGURE 3 | Comparative representation of relative abundance of fecal microbiota at the family level in young-adult and old mice before and after *Listeria monocytogenes* infection. **(A)** Showing high abundance bacteria (>10%) at the family level. **(B)** Intermediate abundance level (<6%) bacteria at the family level. **(C)** Low level (<2%) bacteria at the family level. Taxonomic composition for the abundant bacteria at the family level was generated from k-mer analyses based on taxonomic profiling. Data from mean ± SEM from a representative experiment using 4–6 mice. **p < 0.01.

to uninfected young-adult mice. Interestingly, *L. monocytogenes* infection significantly ($p<0.05$) increased their abundance only in young-adult mice. We observed marked increases in the abundance of *Porphyromonadaceae* and *Prevotellaceae* family bacteria only in the infected young-adult mice. On the other hand, both *Lachinospiraceae* and *Ruminococcaceae* increased significantly ($p<0.05$) in *Listeria*-infected old mice. We are unsure of the reason that *L. monocytogenes* infection caused a differential increase in the relative abundances in young-adult mice compared to old mice. We also detected an abundance of the *Listeriaceae* family bacteria in feces from infected old mice (**Figure 3C**).

Aging Reduces Specific Genus Level Commensal Bacteria Responsible for Colonization Resistance Against *Listeria monocytogenes* Infection in Mice

We performed centroid classification of fecal microbiota at the genus and species level based on their relative abundance in young-adult and old mice after *L. monocytogenes* infection (**Figure 4**), and identified several commensal bacteria that are often reported to have significant protective roles during infection and/or disease. We detected sixteen species of bacteria in young-adult mice and thirteen species in old mice, with eight species common in between both age groups and their abundances altered after infection. Besides *Lachnopiraceae* family bacteria, which are less abundant in young-adult mice, seven additional bacterial species (*Oscillibacter* sp. 1-3, *Enterococcus faecalis*, *Clostridium* sp. ASF502, *Clostridioides difficile*, *Clostridaceaea*_u_s, *Bacteroides uniformis* and *Akkermansia muciniphila*) were only abundant in the feces of the young-adult mice. On the other hand, *Anearotruncus* sp. (G32012) and *Alistipes*_u_s bacteria were only detected in old mice. *Akkermansia muciniphila* (Phylum *Verrucomicrobia*), a mucin degrading bacterium often associated with a healthy gut were only present in the young-adult mice and barely detected in any of the old mice. Fransel et al. (9) also reported a similar increased abundance of *Akkermansia* in young-adult mice. *Clostridium* sp. ASF 502, *Clostridioides difficili*, *Clostridiales* u_s and *Clostridiaceae* u_s belonging to *Clostridiaceae* and *Clostridiales* groups were only abundant in young-adult mice but not in old-adult mice.

Recently, Becattini et al. (18) showed that several bacterial species including, majority of taxa belonging to the order *Clostridiales*, are associated with protection against *in vivo L. monocytogenes* infection. Interestingly, this group of bacteria are mostly absent in the feces of old mice suggesting that the increased susceptibility to *L. monocytogenes* infection in these

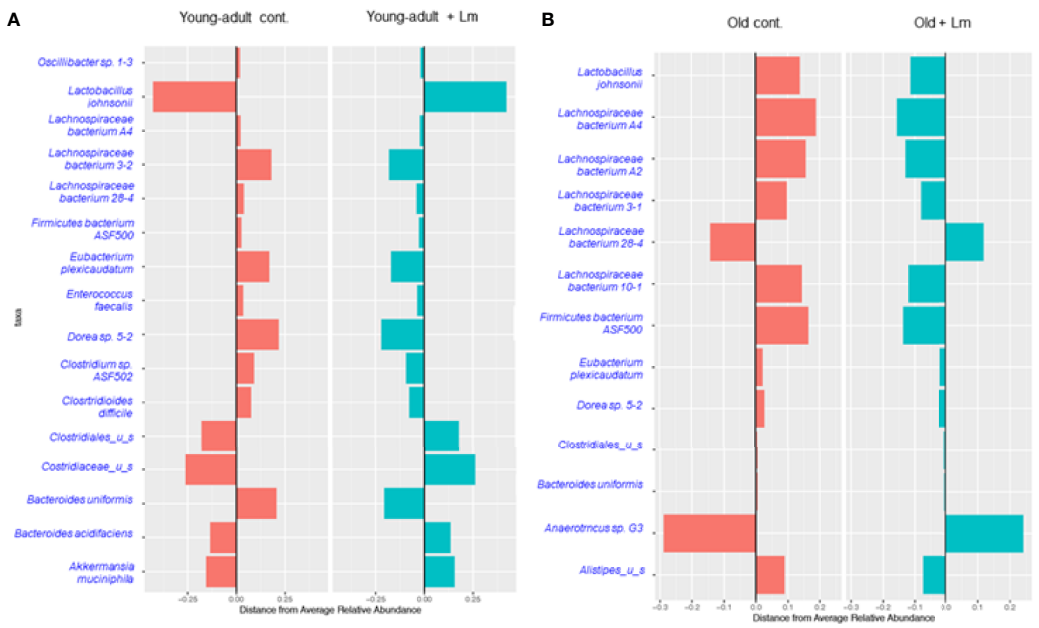

FIGURE 4 | Centroid classification based on relative abundance of fecal microbiota at the genus and species level in young-adult and old mice after *Listeria monocytogenes* (Lm) infection. **(A)** Top sixteen bacterial species that showed either an increase or decrease abundance in young-adult mice before and after *L. monocytogenes* infection. **(B)** Top thirteen frequently bacterial species that showed either an increase or decrease abundance in old mice before and after *L. monocytogenes* infection.

old mice may be due to decreased colonization resistance. We also observed a decreased abundance of *Lactobacillus johnsonii* in the old mice as compared to young-adult mice. Commensals such as *Lactobacilli* spp., are previously known to produce anti-listerial bacteriocins in *in vivo* experiments and were shown to increase resistance to *Listeria* infection in mice with an intact microbiota (31). In general, both species-diversity and abundance were relatively reduced in old mice when compared to young-adult mice suggesting a decreased microbiota diversity or dysbiosis as mice ages.

We also observed an increase in relative abundance and emergence of diverse species of the *Lachnospiraceae* family bacteria, including *Dorea* sp. mostly present in old mice as compared to young-adult mice (**Figure 4**). A least three new *Lachnospiraceae* species (*Lachnospiraceae* bacterium A2, *Lachnospiraceae* bacterium 3-1 and *Lachnospiraceae* bacterium 10-1) were detected in old mice.

Our metagenomic analyses further suggested significant presence of *Parabacteroides_unclassified*, *Prevotella buccae* and *Blautia_unclassified* bacteria in young-adult mice (**Figure 5A**). Both *Parabacteroides_unclassified* and *Prevotella buccae* which belong to *Porphyromonadaceae* and *Prevotellaceae* family respectively, were significantly (p=0.013) increased after infection in young-adult mice On the other hand, *Blautia_unclassified*, which belongs to *Lachnospiraceae* family were significantly (p=0.027) decreased in infected young-adult mice as compared to uninfected mice. It is important to note that, *Blautia* was not detected in old mice. *Alistipes finegoldii*, a commensal bacterium belonging to *Rikenellacdeae* family are exclusively present in the old mice We observed, *Listeria* infection significantly reduced its abundance in old mice (**Figure 5B**).

Pseudoflavonifractor capillosus, *Feacalibacterium* spp. and *Anaerotruncus* sp. G3 (2012) bacteria, all butyrate producers, were identified as highly abundant in infected old mice (**Figures 4B**, **5C**) but the abundance of *Parabacteroides*_unclassified decreased in infected old mice (**Figure 5C**). *Feacalibacterium* spp. and *Anaerotruncus* sp. G3 (2012) species belong to *Ruminococcaceae* family bacteria. As shown before we observed a significant (p<0.05) rise in abundance of *Ruminococcaceae* family bacteria only in the *L. monocytogenes*-infected old mice as compared to *L. monocytogenes*-infected young-adult mice (**Figure 3B**). Our analysis of the metagenomic data associated with bacteria belonging to the *Listeriaceae* family, we were able to detect the *L. monocytogenes* with which our experimental mice were orally infected, confirming persistence of *L. monocytogenes* in the feces on day seven of infection. Fecal samples from only infected-old mice showed higher abundances for *L. monocytogenes* strain (in 40% of the infected old mice with ~100% match to *EGD-e*) (data not shown). We were unable to detect the $InlA^m$ mutation from these samples in our read mapping results, due to the low abundance of reads associated with *Listeria* identified from these samples.

Increased Intestinal Tissue Pathology and Inflammatory Immune-Biomarker Response in the Old Mice

Previously, we showed that old C57BL/6 mice were more susceptible to infection and had significant inflammatory

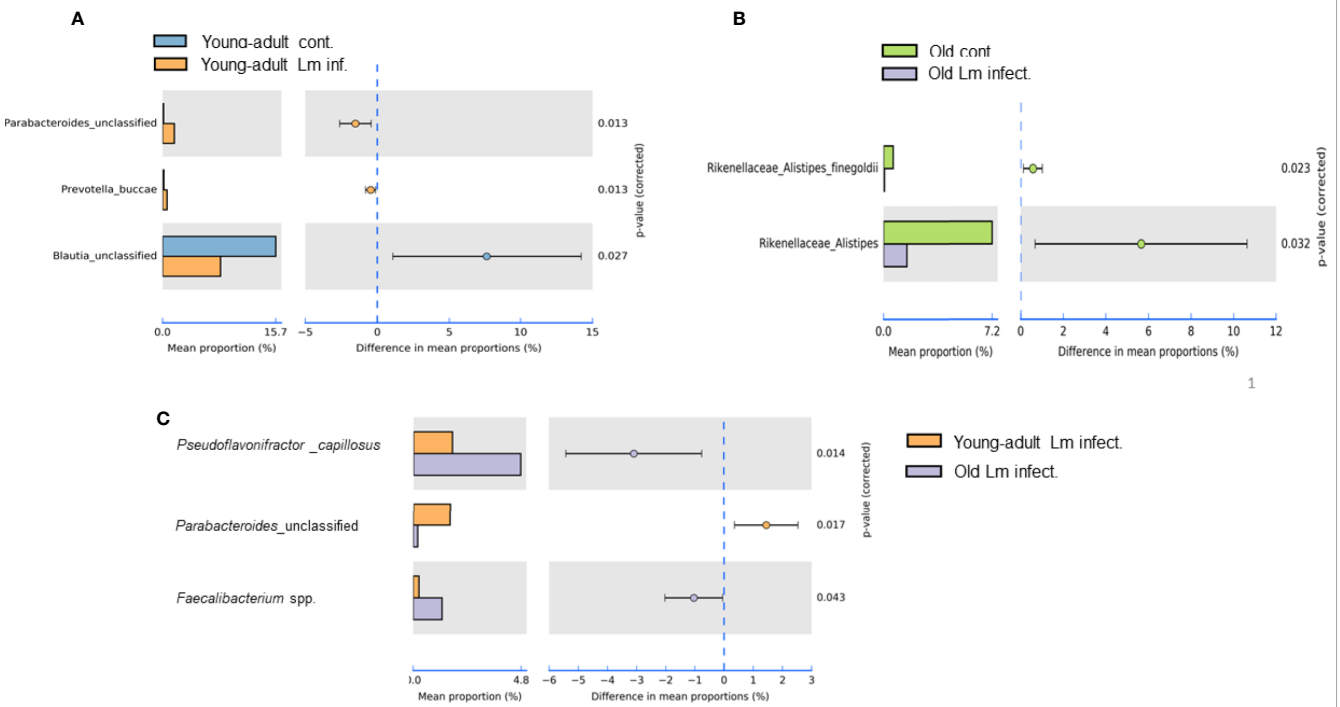

FIGURE 5 | Species level relative abundance of fecal bacteria in young-adult and old mice before and after *Listeria monocytogenes* infection. **(A)** *Listeria*-infection induced changes of bacteria *Parabacteroides*, *Prevotella buccae*, *Blauia* in young-adult mice before and after *Listeria monocytogenes* infection. **(B)** *Listeria*-infection induced changes of bacterial species of the *Rikenellaceae* family that included the genus *Alistipes*, in particular *Alistipes finegoldii*, in old mice before and after *Listeria monocytogenes* infection. **(C)** *L. monocytogenes* infection induced changes in abundance *Pseudoflavonifractor capillosus*, *Parabacteroides*, and *Faecalibacterium* spp. in young-adult and old mice. Relative abundance levels were calculated at the 95% confidence intervals and *p*-value calculated for significance as shown in the figure. The relative abundance of Taxa at the genus level were generated from a k-mer database used for taxonomic profiling. Data from mean ± SEM from a representative experiment using 4–6 mice.

changes in liver and spleen tissues after repeated gavage with *L. monocytogenes* (7). This time, we evaluated gastrointestinal pathology and inflammatory response after *L. monocytogenes* infection. Histologic scoring of gastritis in uninfected and infected young-adult and old mice are shown in **Figure 6**. We found infected older mice had significantly ($p<0.05$) more gastric pathology (**Figures 6B, C**). **Figure 7** shows the gastritis and liver inflammation in old mice as compared to young-adult mice after oral *L. monocytogenes* infection, as analyzed by immunohistochemical evaluation of tissue by myeloperoxidase

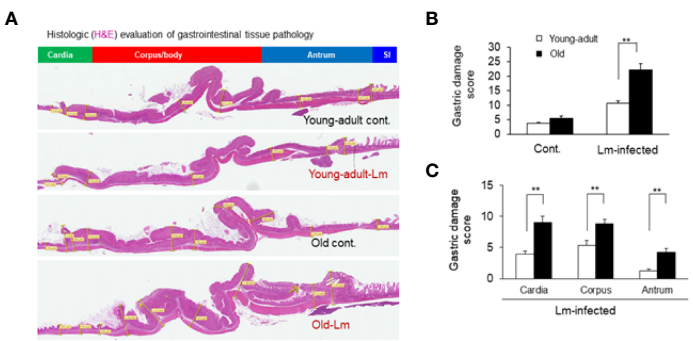

FIGURE 6 | Gastric inflammation was more severe in *Listeria monocytogenes*–infected old mice. Mice were infected by gavage with 1 x 10^6 CFU of *L. monocytogenes* per inoculation for two days, in consecutive, separate inoculations. Mice were euthanized 7-days post infection, and gastric tissue was processed for histologic examination. **(A)** Hematoxylin-eosin–stain of gastric sections from representative control (uninfected) or infected young-adult mice (top two), and control (uninfected) or infected old mice (bottom two). **(B, C)** Histologic scoring of gastritis in uninfected and infected young-adult and old mice. Data from mean ± SEM from a representative experiment using 4–6 mice. **$p < 0.01$.

(MPO) staining (neutrophils). Severe gastritis with dense mononuclear cells (MNC) infiltration and defused MPO-positive granulocytes were noted in the submucosa and mucosa of the old mice in the cardia region. MNC aggregates between the glands spanned the entire width of the mucosa (**Figures 6** and **7**). Intestinal tissues from uninfected and *L. monocytogenes*-infected mice of both age groups were further measured for immune-biomarkers expression (IFN-γ, IL-17a, IL-23 and IL-10 mRNA) (**Figure 8**). *L. monocytogenes* infection increased *in vivo* inflammatory cytokine mRNA responses in the intestinal tissue from old mice which corelated with histological inflammation observed before.

DISCUSSION

Mice are resistant to oral *L. monocytogenes* infection mostly due to mismatch in the species-specificity of pathogen interaction with corresponding host cells receptors (20), and also, in some part, due to commensal intestinal microbiota responsible for colonization resistance (18). We employed a murinized *L. monocytogenes* strain which is capable of breaching murine intestinal epithelial layers when mice are intragastrically infected (6, 20). A previous study demonstrated that aging is linked to an altered gut microbiota composition, inflammation, and increased gut permeability (34). Commensal bacteria can be recognized by the innate immune system and that individual species or groups of commensal bacterial species can influence distinct modules of innate and adaptive immune response (35). Therefore, any dysbiosis of commensal due to aging or infection can modulate immune response.

In our current study, we evaluated if changes in pathological and immune-biomarker response in old mice had any positive-correlation with an altered commensal microbiota population. *Listeria* infection caused mild inflammation in young-adult mice, but more inflammation and pathology in older mice. We also observed altered gut microbiota composition in the old mice prior to infection. In fact, such perturbation or dysbiosis can happen as mice age (9, 36) and infection may further alter that dynamic. As shown in **Figures 6** and **7**, infected older mice had higher gastric pathology, with marked infiltration of polymorphonuclear neutrophil (PMN). We also observed relatively low-level inflammation in old mice even before infection. Our metagenomics data showed that aging resulted in significant dysbiotic changes in the fecal microbiota population, for example, decreased diversity and lower relative abundance of specific anti-listerial species of bacteria, like *Clostridiales* and *Lactobacillus*. Reduced abundance of these

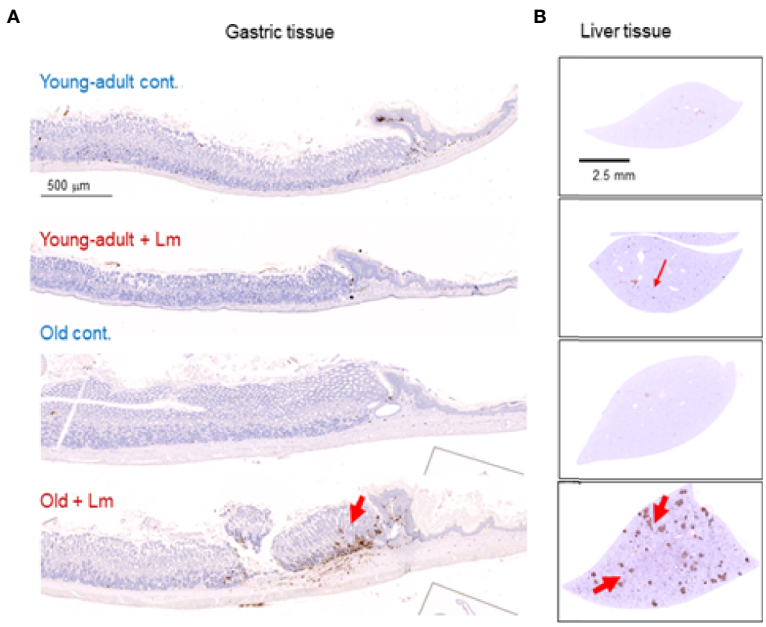

FIGURE 7 | Increased gastritis and liver inflammation in old mice as compared to young-adult mice after oral *Listeria monocytogenes* infection analyzed by immunohistochemical evaluation of gastrointestinal tissue by myeloperoxidase (MPO) staining (neutrophils). **(A)** Myeloperoxidase (MPO) (left column) gastric sections from representative control (uninfected) or infected young-adult mice (top two), and control (uninfected) or infected old mice (bottom two). Arrows denote cells expressing MPO. Only a few scattered mononuclear cell (MNCs) and MPO-positive granulocytes can be seen in the submucosa and lamina propria, with no abnormal thickening of the gastric wall noted in uninfected control mice. Severe gastritis with dense MNC infiltration and defused MPO-positive granulocytes were noted in the submucosa and mucosa of the old mice in the cardia region. MNC aggregates between the glands spanned the entire width of the mucosa. **(B)** Myeloperoxidase (MPO) (right column) liver sections from representative control (uninfected) or infected young-adult mice (top two), and control (uninfected) or infected old mice (bottom two). Arrows denote cells expressing MPO. Data from mean ± SEM from a representative experiment using 4–6 mice.

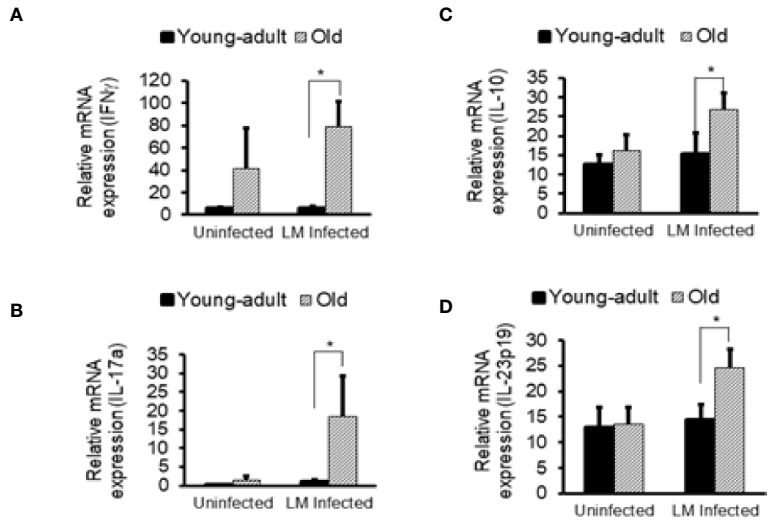

FIGURE 8 | Increased *in vivo* inflammatory cytokine mRNA responses in the intestinal tissue from *Listeria monocytogenes*-infected old mice. Intestinal tissues from uninfected and *L. monocytogenes* (Lm)-infected mice of both age groups at 7-days post infection were used for RNA extraction and IFN-γ **(A)**, IL17a **(B)**, IL-10 **(C)**, and IL-23 **(D)** mRNA expression were measured. Data from mean ± SEM from a representative experiment using 4–6 mice. *$p < 0.05$.

important commensals may compromise colonization resistance in older mice allowing increased *L. monocytogenes* infection through the oral route, intestine and further disseminating into the systemic sites, including liver (**Supplementary Figure 1**).

We also seen the presence of *Parabacteroides_unclassified*, *Prevotella buccae* and *Blautia_unclassified* bacteria in young-adult mice (**Figure 5A**). Both *Parabacteroides_unclassified* and *Prevotella buccae* which belong to *Porphyromonadaceae* and *Prevotellaceae* family respectively, were increased after infection in young-adult mice. The *Porphyromonadaceae* and *Prevotellaceae* families are commensal and associated with inflammatory response to infection (37, 38). The abundance of *Parabacteroides_unclassified* decreased in infected old mice (**Figure 5C**) and those mice also had increased anti-inflammatory (IL-10) response. On the other hand, *Blautia_unclassified*, which belongs to *Lachnospiraceae* family were significantly decreased in infected young-adult mice as compared to uninfected mice. Murri et al. (39) reported that *Blautia* abundance is associated with a healthy gut microbiome and their abundance can decrease during human liver diseases and in certain cancers, which may be correlated with its significant reduction in infected-young-adult mice. It is important to note that, *Blautia* was not detected in old mice. *Alistipes finegoldii*, a commensal bacterium belonging to *Rikenellacdeae* family are exclusively present in the old mice and believed to be indicative of gastrointestinal health. Its abundance in the gut has shown to be decreased during gastrointestinal inflammation (36). We observed, *Listeria* infection significantly reduced its abundance in old mice (**Figure 5B**) that may correlate with increased gastric inflammation seen in these mice (**Figures 6** and **8**). As shown before *Pseudoflavonifractor capillosus*, *Feacalibacterium* spp. and *Anaerotruncus* sp. G3 (2012) bacteria, all butyrate producers, were identified as highly abundant in infected old mice, and have been reported to be involved in the regulation of inflammation response during human diseases and infection (34, 36). We observed a significant rise in abundance of these bacteria only in the *L. monocytogenes*-infected old mice as compared to *L. monocytogenes*-infected young-adult mice (**Figure 5C**). Both *Feacalibacterium* spp. and *Anaerotruncus* sp. G3 (2012) species belong to *Ruminococcaceae* family bacteria. One report suggested that *Feacalibacterium prausnitzii* A2-165 strain can induce IL-10 production in dendritic cells and modulate T cell response (40). Notably, our study also showed similar increased anti-inflammatory (IL-10) response (**Figure 8C**) in old infected mice which may correlate the increased abundance of members of the *Ruminococcaceae* family bacteria in the feces from old mice (**Figure 3B**).

The *Lachnospiraceae* family are more abundant in infected old mice (**Figure 3B**), and have been reported to be associated with inflammation and obesity (41, 42); this data correlates with our findings that show a higher inflammatory response in infected old mice (**Figures 6–8**). Our histology data suggest that *L. monocytogenes*-infected old mice had a significantly heightened level of inflammatory biomarkers which may correlate with increased diversity of the *Lachnospiraceae* family member bacteria. In addition, the family *Lachnospiraceae* has been reported to have a possible anti-inflammatory role (43, 44), but its specific role has yet to be elucidated. Previously in our study, we reported an increased anti-inflammatory response (IL-10 and Treg cells) in the old mice (7). Furthermore, we observed an increased relative abundance of *Firmicutes* bacterium ASF500 species belonging to phylum *Firmicutes* in old mice as compared to young-adult mice. Interestingly, Atarashi et al. (45) showed that the *Firmicutes* bacterium ASF500 can induce pro-inflammatory IL-17 cells which we also observed in our previous study. Notably, in our current study, intestinal IL-17

and IL-23 mRNA response from infected old mice was also high (**Figures 8B, D**).

The common bacteria in the phylum *Firmicutes*, including the *Clostridium* cluster XIVa, take part in predominant role in the fermentation of carbohydrates within the gut (46). The crucial end products of this fermentation in the gut are various short-chain fatty acids (SCFAs) like, acetate, propionate, and butyrate. *Firmicutes* is the principal bacterial phylum, containing over 250 genera, including *Lactobacillus*, and *Clostridium* which can generate several SCFAs, including butyrate. Butyrate serves as the main source of nutrition for cells of the gut epithelium (47, 48). Depletion, or any change of butyrate levels, is associated with inflammation and impairments in the gut barrier integrity (49, 50). *Akkermansia muciniphila*, a mucin degrading bacterium in the gut is only found in the young-adult mice and not detected in old mice. We did not study the cause-and-effect relationship of any these bacteria mentioned, on their role in inflammatory response *per se*, but suggest that these species could be modulating inflammation *via* SCFAs, including butyrate production as reported by other studies (43, 48). Our results suggest that the decreased proinflammatory response (IFN-γ) and increased anti-inflammatory (IL-10) response we observed in our earlier study in old mice may be, in part, due to an increased abundance of *Lachonospiraceae*. In our current study, we observed increased neutrophil infiltration in intestinal tissue and liver in the Lm-infected old mice that could be due to SCFAs, derived from dysbiosis of commensals. It is now well recognized that SCFAs can regulate immune cells. SFCAs, propionate and acetate, derived from commensal bacteria, promote neutrophil chemotaxis (51) and further focused studies are warranted.

Conclusion

Foodborne *L. monocytogenes* infection is a public health problem, especially in the susceptible populations (elderly, pregnant, and immune-compromised person). We developed a surrogate mice model that mimics human foodborne listeriosis and investigated the role of gut-microbiota correlating with immune-status on the risk of developing listeriosis. We have shown that aging alters gut microbiota composition and may compromise colonization resistance against *L. monocytogenes* infection. In older mice, species-diversity and abundance is reduced. Specifically, beneficial commensal like *Lactobacilus* spp., and taxa belonging to the order *Clostridiales* are reduced or completely absent in older mice. We hypothesize infection with *L. monocytogenes* in older mice may facilitate increased numbers of specific immune-modulating bacteria belonging to the *Lachnospiraceae* and *Ruminococcaceae* families. *L. monocytogenes* infection in mice can cause marked perturbation of the host gut microbiota and a recent report by Rolhion et al. (52) showed that bacteriocin from *L. monocytogenes* can target the commensal *Prevotella copri* and modulate intestinal infection. We measured tissue inflammation response of young-adult and old mice before and after infection and correlated that response to dysbiosis of commensal bacteria due to aging. We did not study the functional relations/response as to why aging or infection altered the gut microbiota composition and how that can affect immune function. Our study is rather limited to establishing a possible correlation between dysbiosis with increased risk of listeriosis under aging condition. Also, it is still unclear whether the dysbiosis is a cause or consequence of inflammation. We propose that aging may cause significant dysbiosis of commensal microbiota in older mice that may compromise their immune balances. In addition, with the loss of beneficial anti-listerial commensal bacteria, increased *L. monocytogenes* colonization in the gut can occur, that may further perturb immune-modulating bacteria that are responsible for plethora of immune activation resulting in increased risk and disease severity.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA691798.

ETHICS STATEMENT

The protocol (protocol approval number: BFQ-11-006) was approved by the Food and Drug Administration, Center for Food Safety and Applied Nutrition-Institutional Animal Care and Use Committee (CFSAN-IACUC). All mice were kept at the MOD-1, CFSAN/FDA, AAALAC (American Association for Accreditation of Laboratory Animal Care) accredited animal facility.

AUTHOR CONTRIBUTIONS

MA and CT conceived and designed the research study, provided the administrative oversight, and wrote the manuscript. MA, CT, NH, JG and TB performed the experiments, and analyzed the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.672353/full#supplementary-material

Supplementary Figure 1 | Increased systemic dissemination of *Listeria monocytogenes* (Lm) in old mice after oral infection on 7-days post infection. **(A, B)** Viable *L. monocytogenes* colony count in spleen and liver tissues on 7-dpi. **(C)** Increased colonization of *L. monocytogenes* (green, FITC labeled) in liver tissue detected by immuno-staining with FITC-anti-Lm antibody. Data from mean ± SEM from a representative experiment using 4–6 mice. $^{*}p < 0.05$, $^{**}p < 0.01$.

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frontiers in Immunology

ORIGINAL RESEARCH
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Trans Fatty Acid Intake Induces Intestinal Inflammation and Impaired Glucose Tolerance

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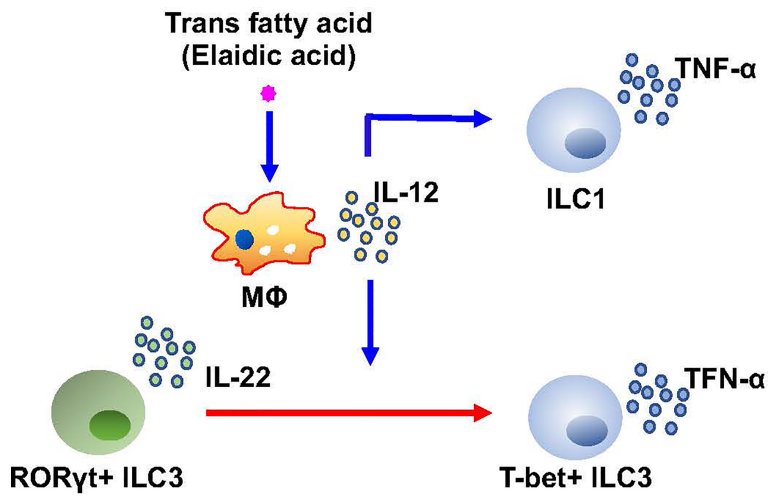
Background and Aims: Many nutritional and epidemiological studies have shown that high consumption of trans fatty acids can cause several adverse effects on human health, including cardiovascular disease, diabetes, and cancer. In the present study, we investigated the effect of trans fatty acids on innate immunity in the gut by observing mice fed with a diet high in trans fatty acids, which have been reported to cause dysbiosis.

Methods: We used C57BL6/J mice and fed them with normal diet (ND) or high-fat, high-sucrose diet (HFHSD) or high-trans fatty acid, high-sucrose diet (HTHSD) for 12 weeks. 16S rRNA gene sequencing was performed on the mice stool samples, in addition to flow cytometry, real-time PCR, and lipidomics analysis of the mice serum and liver samples. RAW264.7 cells were used for the *in vitro* studies.

Results: Mice fed with HTHSD displayed significantly higher blood glucose levels and advanced fatty liver and intestinal inflammation, as compared to mice fed with HFHSD. Furthermore, compared to mice fed with HFHSD, mice fed with HTHSD displayed a significant elevation in the expression of CD36 in the small intestine, along with a reduction in the expression of IL-22. Furthermore, there was a significant increase in the populations of ILC1s and T-bet-positive ILC3s in the lamina propria in mice fed with HTHSD. Finally, the relative abundance of the family *Desulfovibrionaceae*, which belongs to the phylum *Proteobacteria*, was significantly higher in mice fed with HFHSD or HTHSD, than in mice fed with ND; between the HFHSD and HTHSD groups, the abundance was slightly higher in the HTHSD group.

Conclusions: This study revealed that compared to saturated fatty acid intake, trans fatty acid intake significantly exacerbated metabolic diseases such as diabetes and fatty liver.

Keywords: innate lymphoid cells, ILC, trans fatty acid, gut microbiota, small intestine



Q7 **GRAPHICAL ABSTRACT** | Trans-fatty acids (elaidic acids) act on macrophages in the lamina propria of small intestine and liver to stimulate the secretion of IL-12, which not only promotes the secretion of TNF-α from ILC-1, but also causes a stronger inflammatory response by changing RORγt-positive ILC3 to T-bet-positive ILC3 with ILC1-like effects.

HIGHLIGHTS

In this study, we investigated the effect of trans fatty acids on innate immunity in the gut by comparing mice fed with a diet high in trans fatty acids with mice fed with a normal diet or a diet high in saturated fatty acids. We revealed that trans fatty acid intake significantly exacerbated metabolic diseases such as diabetes and fatty liver when compared to saturated fatty acid intake. It has been suggested that this is mainly due to inflammatory modification of the innate immunity, in particular, an increase in the number of intestinal T-bet-positive ILC3s and a significant decrease in the production of IL-22 in the intestine and liver.

INTRODUCTION

Trans fatty acids are a generic term for artificial fatty acids containing trans carbon-carbon double bonds, which are mainly produced in the food production process. Margarine or shortening, a hardened oil produced by hydrogenation, is a typical food with trans fatty acids. Humans now consume trans fatty acids in a way that was not part of our diet in the past. Many nutritional and epidemiological studies have shown that high consumption of trans fatty acids can cause several adverse effects on human health, including cardiovascular disease, diabetes, and cancer (1).

The gut of humans and other mammals contains trillions of microorganisms, which are collectively known as the gut microbiota. The gut microbiota functions as an endocrine organ, helping to shape the intestinal immune response and produce metabolites that are involved in many aspects of the normal host physiology (2). The gut microbiota provides important benefits to the host, especially for metabolic and immune development; thus, homeostasis of the gut microbiota is important for maintaining the health of the host (3). As a result, abnormal changes in the composition and biodiversity of the gut microbiota, known as dysbiosis, can be an important cause of several metabolic syndromes, such as inflammatory bowel disease (IBD), obesity, dyslipidemia, diabetes, heart disease, and cancer (4–8), mediated through chronic inflammation and insulin resistance (4, 8). It is widely accepted that diet is a major factor that regulates the composition of the gut microbiota in humans and mice (7).

In the past decade, a group of lymphocytes, which act in innate immunity and do not express antigen receptors, has been discovered and named innate lymphoid cells (ILCs). Currently, ILCs are classified into three groups: ILC1, ILC2, and ILC3. ILC1s produce IFN-γ to protect against intracellular bacteria and viruses through activation of macrophages. The transcription factor T-bet is involved in the induction of ILC1 differentiation (type 1 immune response). ILC3s produce interleukin (IL)-17 and IL-22, which are involved in the defense against extracellular bacteria and fungi through mobilization of neutrophils, as well as, activation and proliferation of epithelial cells.

In the present study, we hypothesized that trans fatty acids cause dysbiosis and associated immune changes in the intestine. The purpose of this study was to confirm the effect of trans fatty acids on innate immunity in the gut by comparing mice fed with a diet high in trans fatty acids [which have been reported to cause dysbiosis (9)] with mice fed with a normal diet or a diet high in saturated fatty acids.

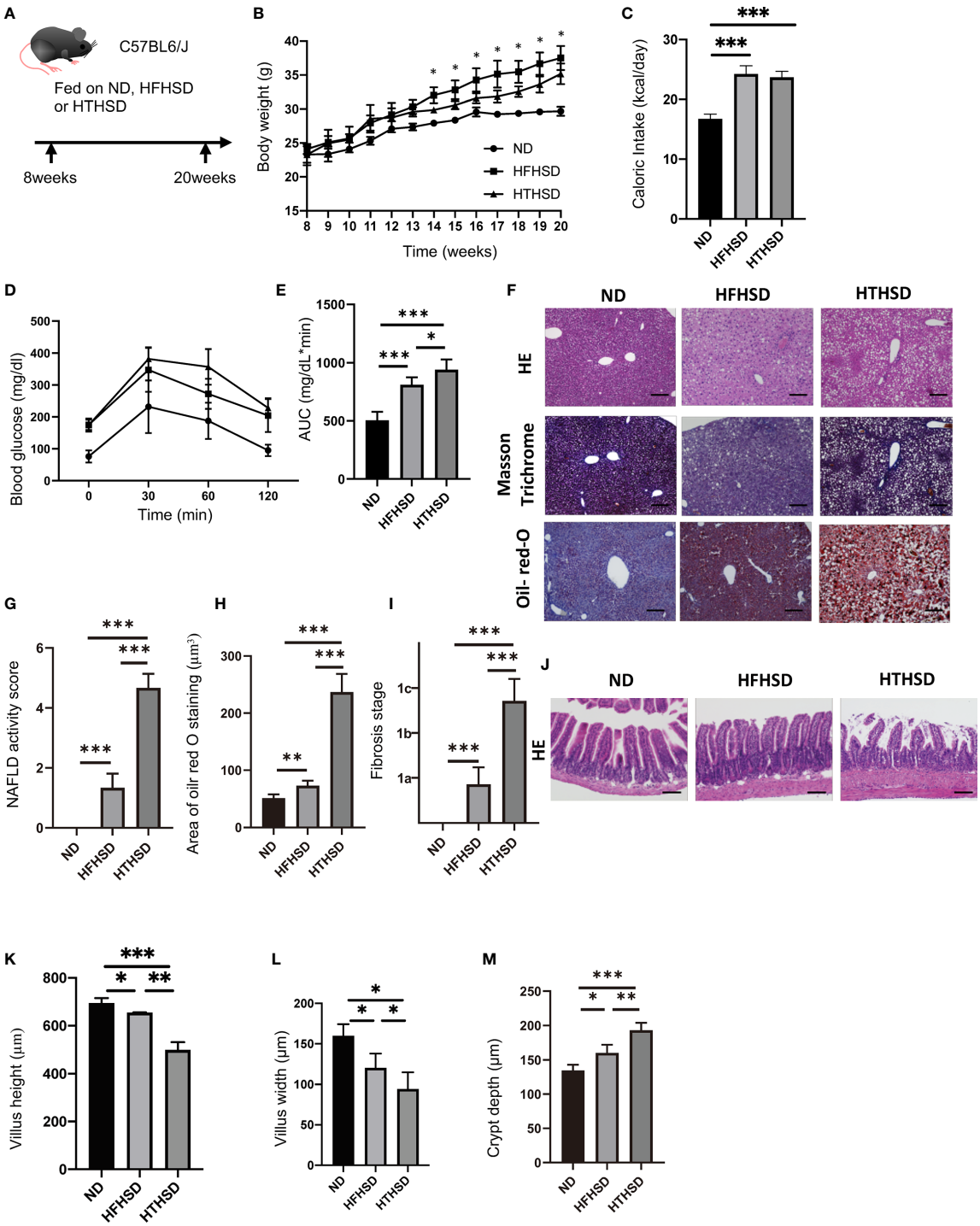


FIGURE 1 | Trans fatty acids significantly worsen glucose tolerance, despite causing only mild weight gain, as compared to saturated fatty acids. **(A)** Eight-week-old C57BL6/J mice were fed with ND, HFHSD, or HTHSD for 12 weeks, starting at 8 weeks of age. The mice were sacrificed when they reached 20 weeks of age. **(B)** Changes in the body weights of the mice (n=6). If the weight of mice fed with HFHSD was significantly higher than that with HTHSD by an unpaired, two-tailed Student's t test, it was marked with asterisk on top. **(C)** Caloric intake measured at 20 weeks of age (n=6). **(D, E)** When the mice reached 20 weeks of age, an intraperitoneal glucose tolerance test (2 g/kg body weight) was performed, followed by analysis of the area under the curve (n=6). **(F)** Representative liver histology of the mice. **(G)** NAFLD activity score (n=6). **(H)** Oil Red O-stained area (n=6). **(I)** Fibrosis stage (n=6). **(J)** Representative jejunum histology of the mice. **(K)** Villus height (n=6). **(L)** Villus width (n=6). Data are represented as mean ± SD; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ using Tukey HSD test. ND, normal diet; HFHSD, high-fat, high-sucrose diet; HTHSD, high-trans fatty acid, high-sucrose diet. **(M)** Crypt depth (n=6).

MATERIALS AND METHODS

Animals

All experimental procedures were approved by the Committee for Animal Research at the Kyoto Prefectural University of Medicine. Seven-week-old C57BL/6J (wild-type) male mice were purchased from Shimizu Laboratory Supplies (Kyoto, Japan) and housed in a specific pathogen-free controlled environment. We used the littermate mice that were born at the same time at a mouse supply facility. Moreover, we housed weight-matched 10 animals in two cages (5 animals per cage) in each group, experimented with all of them, and took data from a total of six animals, excluding the two larger mice and smaller mice at sacrifice. The mice were fed a normal diet (ND; 345 kcal/100 g, fat kcal 4.6%; CLEA, Japan, Tokyo), or high-fat, high-sucrose diet (HFHSD; 459 kcal/100 g, 20% protein, 40% carbohydrate, and 40% fat, coconut oil; D12327, Research Diets Inc., New Brunswick, NJ, USA), or high-trans fatty acid, high-sucrose diet (HTHSD; 459 kcal/100 g, 20% protein, 40% carbohydrate, and 40% fat, shortening; D18120301, Research Diets Inc.) for 12 weeks, starting at 8 weeks of age. Body weights of the mice were measured every week. When the mice reached 20 weeks of age, they were sacrificed by administration of a combination anesthetic with 0.3 mg/kg medetomidine, 4.0 mg/kg midazolam, and 5.0 mg/kg butorphanol, after an overnight fast (10) (**Figure 1A**).

Measurement of Caloric Intake

The oral intake of the mice was measured at 20 weeks. Mice were housed individually and weighed food was placed in a trough in each cage. After 24 h, the amount of remaining food was weighed. Caloric intake was calculated by subtracting the final amount of food from the initial amount of food.

Analytic Procedures for Glucose and Insulin Tolerance Tests

An iPGTT (2 g/kg of body weight) was performed in 20-week-old mice that were made to fast for 16 h. The blood glucose levels were measured from drops of blood at the time-points indicated, using a glucometer (Gultest Neo Alpha; Sanwa Kagaku Kenkyusho, Nagoya, Japan). The results of the iPGTT test were analyzed by measuring the AUC.

Protocol for Isolation of Mononuclear Cells From Livers and Small Intestines of Mice

To exclude blood contamination in the liver and small intestine, systemic perfusion with heparinized saline was performed before harvesting or washing the liver and small intestinal tissues with phosphate-buffered saline (PBS). The liver was perfused with 20 mL of PBS (pH 7.0) and then harvested. Isolation of hepatic lymphocytes by mechanical dissection was performed using the methods described in previous studies (11). Briefly, a suspension of liver tissue in Roswell Park Memorial Institute (RPMI) 1640 medium containing 20 mL/L fetal bovine serum (FBS, 2%) was centrifuged. The obtained pellet was resuspended in 40% Percoll® solution, layered on top of an equal volume of 60% Percoll® solution and centrifuged, followed by extraction of the middle layer.

Intestinal lamina propria mononuclear cells were isolated according to the instructions of the Lamina Propria Dissociation Kit (130-097-410; Miltenyi Biotec, Germany). Cell pellets were resuspended in 40% Percoll® and added slowly to the upper portion of the centrifuge tubes, which contained 5 mL of 80% Percoll® at the bottom. Lamina propria mononuclear cells were obtained by washing twice with 2% FBS/PBS, post density gradient centrifugation at 420 × *g* for 20 min.

Tissue Preparation and Flow Cytometry

Stained cells were analyzed on a FACSCanto™ II system and the data were analyzed using FlowJo software version 10 (TreeStar, Ashland, OR, USA). The innate lymphoid cells were gated using methods provided in a previous study (11, 12), with antibodies described in the supplementary file.

Liver Histology

Liver tissue was obtained and fixed with 10% buffered formaldehyde or embedded in paraffin. Tissue sections were prepared and stained with hematoxylin and eosin (H&E) and Masson's Trichrome stains. Additionally, the non-alcoholic fatty liver disease (NAFLD) activity score was adopted to assess NAFLD severity, as in our previous study (13), the details for which are provided in the supplementary file.

The liver sections were also subjected to Oil Red O staining. The tissues were fixed in 4% paraformaldehyde overnight at 4°C. Liver tissues, which were frozen in OCT compounds, were cut into 4 μm-thick sections, mounted onto slides, and allowed to dry for 1–2 h. The sections were then rinsed with PBS, pH 7.4. After air drying, the slides were placed in 100% propylene glycol for 2 min and stained with 0.5% Oil Red O solution in propylene glycol for 30 min. The slides were transferred to an 85% propylene glycol solution for 1 min, rinsed in distilled water for two changes, and processed for hematoxylin counterstaining.

Images were captured with a fluorescence microscope BZ-X710 (Keyence, Osaka, Japan), followed by analysis of the Oil Red O-stained area of the liver tissue using ImageJ software.

Small Intestine Histology

The small intestine tissue was obtained and either fixed with 10% buffered formaldehyde or embedded in paraffin. Tissue sections were prepared and stained with H&E. Images were captured with a fluorescence microscope BZ-X710. The height/width of the villus and crypt depth were analyzed using ImageJ software.

Gene Expression in Murine Liver and Small Intestine

We used real-time reverse transcription polymerase chain reaction to quantify the mRNA expression levels of *Tnfa, Il6, Il1b*, *Cd36*, *Ccl2*, and *Il22* using the same methods as in our previous study (11), the details for which are provided in the supplementary file. The primer sequence information was provided in the **Supplementary Table**.

Measurement of Fatty Acid Concentrations in the Liver Tissue and Serum Samples

The fatty acid composition of the murine liver and serum samples was analyzed using gas chromatography-mass spectrometry on an Agilent 7890B/5977B System (Agilent Technologies, Santa Clara, CA, USA). Liver tissue (15 μg) and serum (25 μL) samples were methylated using a Fatty Acid Methylation Kit (Nacalai Tesque, Kyoto, Japan). The final product was loaded onto a Varian capillary column (DB-FATWAX UI; Agilent Technologies). The capillary column used for fatty acid separation was CP-Sil 88 for FAME (100 m × an inner diameter of 0.25 mm × membrane thickness of 0.20 μm, Agilent Technologies). The column temperature was maintained at 100°C for 4 min, increased gradually by 3°C/min to 240°C, and then held there for 7 min. The sample was injected in split mode with a split ratio of 5:1. Each fatty acid methyl ester was detected in the select ion-monitoring mode. All results were normalized to the peak height of the C17:0 internal standard (14).

Murine Macrophage Cell Culture and Flow Cytometry

Murine macrophage cells (RAW264.7, KAC Co. Ltd., Kyoto, Japan) were seeded into 24-well plates and grown in DMEM supplemented with 10% FBS. RAW264.7 cells were treated with ethanol, 200 μM palmitic acid or elaidic acid for 24 h. Following that, RAW264.7 cells were pre-treated with phorbol myristiric acid (at the indicated concentrations) for 20 min, prior to stimulation with 1 μM ionomycin for cytokine release.

Stained cells were analyzed using FACSCanto™ II system and the data were analyzed using FlowJo software version 10. Antibodies for gating of *il-12*- and *il-1β*-positive cells were the same as those used in our previous study (11, 15), the details for which are provided in the supplementary file.

Fecal Microbiota Analysis

The fecal samples were collected from the appendix and placed in a cryotube. Immediately afterwards, they were attached to liquid nitrogen for cryopreservation and kept in liquid nitrogen until DNA extraction. Each three fecal samples collected from the appendix of three mice one by one, excluding the one larger mice and smaller mice in a cage of each group. Microbial DNA was extracted from frozen fecal samples using the QIAamp® DNA Stool Mini Kit (Qiagen, Venlo, Netherlands), following the manufacturer's instructions. The PCR reaction was performed using EF-Taq (SolGent, Korea), with 20 ng of genomic DNA as a template in a 30 μL reaction mixture. The PCR protocol included activation of Taq polymerase at 95°C for 2 min, followed by 35 cycles at 95°C, 55°C, and 72°C for 1 min each, finished with a 10 min step at 72°C. The amplified products were purified using a multiscreen filter plate (Millipore Corp., Billerica, MA, USA). Sequencing was performed using a BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). DNA samples containing the extension products were added to Hi-Di™ formamide (Applied Biosystems). The mixture was incubated at 95°C for 5 min, followed by 5 min on ice, and then analyzed using an ABI Prism 3730xl DNA Analyzer (Applied Biosystems). 16S rRNA sequencing was performed by Macrogen (Seoul, Korea). QIIME version 1.9.1 was used to filter sequences for quality (16). Scores less than 75% and mismatches in the barcode or primers were eliminated from the files. The number of operational taxonomic units (OTUs) was determined using the UCLUST algorithm at 97% similarity (17). Taxonomic assignment of 16S rRNA was performed with the Greengenes core-set-aligned with UCLUST and the UNITE sequence sets for ITS using BLAST (UNITE, 2017). A total of 2,873 OTUs of the 16S rRNA gene and 919 ITS OTUs were used in the subsequent analysis.

The relative abundance of the phenotypic categories of the taxonomic groups was predicted using METAGENassist, a statistical tool for comparative metagenomics (18). Data filtering was based on interquantile range, row normalization by sum, and column normalization based on autoscaling. Differences in microbial communities between three groups were investigated using the phylogeny-based unweighted UniFrac distance metric, and principal coordinate analysis (PCoA) plots and beta-diversity with permutational multivariat analysis of variance (PERMANOVA) test with Tinn-R Gui version 1.19.4.7, R version 1.36 (19). The relative abundance of the bacterial genera between the three groups was evaluated using one-way analysis of variance followed by FDR corrections, and that between two groups was evaluated using the weighted average differences (WAD) method with Tinn-R Gui version 1.19.4.7, R version 1.36 (20), and paired an unpaired, two-tailed Student's t test with JMP (SAS Institute Inc., Cary, NC, USA). In this WAD method, the genera were ranked by comprehensively assessing higher expression, higher weight, and fold change. WAD was found to be an effective transcriptome analysis. The relative abundance of functional profiles for the gut microbiota in the groups was evaluated using the WAD method and an unpaired, two-tailed Student's t tests. The top 20 microbial genera in the gut microbiota were determined with the WAD algorithm using R between two groups or small q values evaluated by one-way ANOVA between three groups.

Statistical Analysis

Differences between two groups were assessed using an unpaired, two-tailed Student's t test and Mann-Whitney U test for parametric and non-parametric continuous values, respectively. Differences in categorized variables between two groups were assessed using the Pearson's chi-square test. Differences in continuous variables among more than three groups were assessed using one-way ANOVA. We used Prism software version 8.0 (GraphPad, San Diego, CA, USA). The difference was considered statistically significant at $P<0.05$.

RESULTS

HTHSD Induces Significant Impaired Glucose Tolerance Without Weight Gain

The body weights of mice fed with HFHSD or HTHSD were significantly higher than those of mice fed with ND. Upon

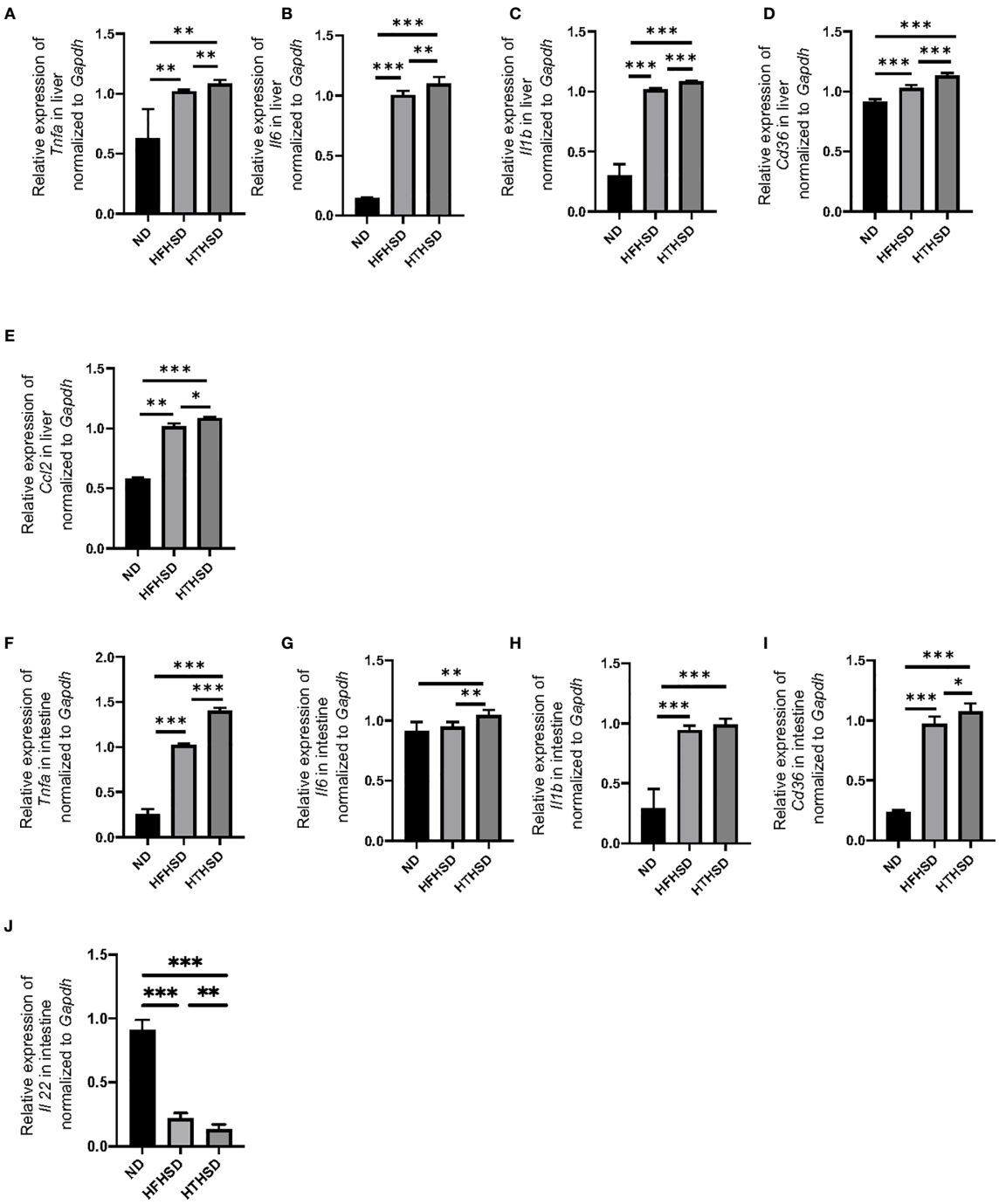


FIGURE 2 | Mice fed with HTHSD displayed significantly elevated inflammation-related gene expression, than mice fed with HTHSD. Gene expression (n=6) in the liver for: **(A)** *Tnfa*, **(B)** *Il6*, **(C)** *Il1b*, **(D)** *Cd36*, and **(E)** *Ccl2*. Gene expression (n=6) in the small intestine for: **(F)** *Tnfa*, **(G)** *Il6*, **(H)** *Il1b*, **(I)** *Cd36*, and **(J)** *Il22*. Data are represented as mean ± SD; *p < 0.05, **p < 0.01, ***p < 0.001 using one-way ANOVA and an unpaired, two-tailed Student's t test. ND, normal diet; HFHSD, high-fat, high-sucrose diet; HTHSD, high-trans fatty acid, high-sucrose diet.

comparing HTHSD and HFHSD groups, body weights of the mice were found to be significantly higher in the HFHSD group, from 14 weeks of age (**Figure 1B**). Among the three groups, the caloric intake of the ND group was significantly lower than that of the HFHSD or HTHSD groups, at 20 weeks of age (**Figure 1C**). In the iPGTT, the AUC of blood glucose increased in the following order of the groups - ND, HFHSD, and HTHSD (**Figure 1D, E**).

HTHSD Aggravates NAFLD and Induces Small Intestinal Inflammation

Both hepatic fat accumulation (**Figures 1F–H**) and liver fibrosis (**Figure 1I**) worsened in the following order of the groups - ND, HFHSD, and HTHSD.

Upon histological analysis of the small intestine, villus height and width reduced in the following order of the groups - ND, HFHSD, and HTHSD (**Figures 1J–L**). Conversely, crypt depth increased in the following order of the groups - ND, HFHSD, and HTHSD (**Figure 1M**).

HTHSD Induces Inflammation and Activation of Fatty Acid Transporter in the Liver and Small Intestine

Next, the relative expression of genes related to inflammation and fatty acid transporters in the liver and small intestine was investigated. Expression levels of *Tnfa, Il6, Il1b*, and *Cd36* in the liver increased in the following order of the groups - ND, HFHSD, and HTHSD (**Figures 2A–D**). Moreover, expression of *Ccl2* increased in the following order of the groups - ND, HFHSD, and HTHSD (**Figure 2E**). Likewise, mice fed with ND, HFHSD, and HTHSD diets displayed increasing expression of *Tnfa* and *Cd36*, with decreasing expression of *Il22* in the small intestine, in that order. Mice fed with HTHSD showed higher expression of *Il-6* in the small intestine than mice fed with ND or HFHSD, and the expression of *Il1b* in mice fed with HFHSD or HTHSD was significantly higher than that with ND, whereas that is not different between mice fed with HFHSD and HTHSD (**Figures 2F–J**). Overall, in both the liver and small intestine, the expression levels of inflammation markers and fatty acid transporters were significantly higher in mice fed with HTHSD, than in those fed with HFHSD.

HTHSD Induces an Increase in the Number of ILC1s, ILC3s, and M1 Macrophages in the Liver

Changes in the number of ILCs and macrophages in the liver were examined using flow cytometry (**Supplementary Figures 1, 2**). ILC1s and ILC3s, which have been reported to contribute to the onset of NAFLD [26–27], were significantly higher in the

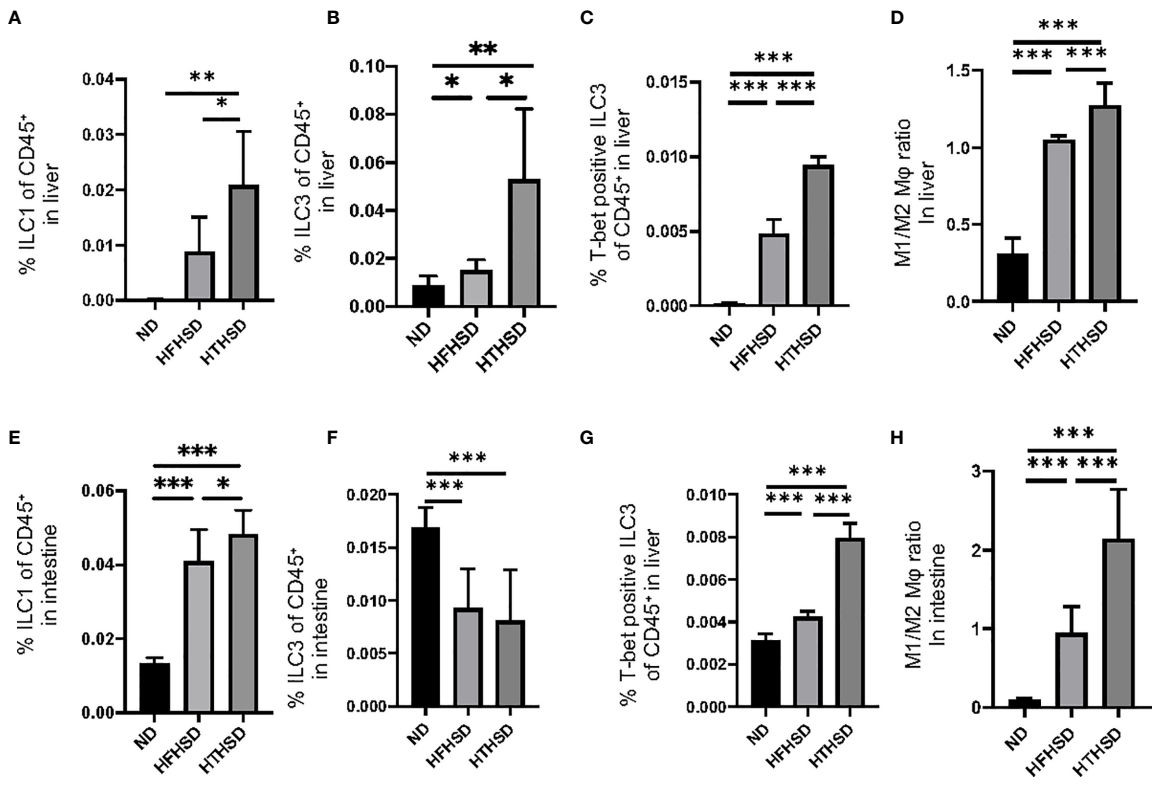


FIGURE 3 | ILC1s, T-bet-positive ILC3s, and M1/M2 macrophages ratio in liver and small intestine in mice fed with HTHSD significantly increased compared. On the other hand, ILC3s in liver in mice fed with HTHSD increased, whereas those in small intestine decreased. **(A)** Ratio of ILC1s to CD45-positive cells in the liver (n=6). **(B)** Ratio of ILC3s to CD45-positive cells in the liver (n=6). **(C)** Ratio of T-bet-positive ILC3s to CD45-positive cells in the liver (n=6). **(D)** Ratio of M1 macrophages to M2 macrophages in the liver (n=6). **(E)** Ratio of ILC1s to CD45-positive cells in the small intestine (n=6). **(F)** Ratio of ILC3s to CD45-positive cells in the small intestine (n=6). **(G)** Ratio of T-bet-positive ILC3s to CD45-positive cells in the liver (n=6). **(H)** Ratio of M1 macrophages to M2 macrophages in the liver (n=6). Data are represented as mean ± SD; *p < 0.05, **p < 0.01, ***p < 0.001 using one-way ANOVA and an unpaired, two-tailed Student's t test.

livers of mice fed with HTHSD, as compared to mice fed with ND or HFHSD (ILC1s, ND: p=0.009 and HFHSD: p=0.029; ILC3s, ND: p=0.043 and HFHSD: p=0.003) (**Figures 3A, B**). In addition, the number of T-bet-positive ILC3s, which secrete high amounts of IFN-γ [28], were found to increase in the following order of the groups - ND, HFHSD, and HTHSD (**Figure 3C**). There are two main phenotypes of macrophages: M1 pro-inflammatory macrophages and M2 anti-inflammatory macrophages [29]. The M1/M2 macrophage ratio, which indicates an association with liver fibrosis [30], increased in the following order of the groups - ND, HFHSD, and HTHSD (**Figure 3D**).

ILC1s in the lamina propria of the small intestine were higher (both p<0.001) in the HFHSD and HTHSD groups than in the ND group; between the HTHSD or HFHSD groups, ILC1 numbers were significantly higher (p=0.045) in the HTHSD group (**Figure 3E**). In addition, ILC3s in the lamina propria of the small intestine were lower (both p<0.001) in the HFHSD or HTHSD groups than in the ND group; in addition, there was no significant difference (p=0.550) between the HFHSD and HTHSD groups (**Figure 3F**).

There was an increase in the number of T-bet-positive ILC3s and the M1/M2 macrophage ratio in the lamina propria of the small intestine, in the following order of the groups - ND, HFHSD, and HTHSD (**Figures 3G, H**).

Elaidic Acid, Which Increases Upon HTHSD Intake, Induces Inflammation

Next, lipidomes in the liver and sera samples were investigated. Among the three groups, palmitic acid concentration was the highest in the liver and sera samples of mice fed with HFHSD, whereas elaidic acid concentration was the highest in the liver and sera samples of mice fed with HTHSD (**Figures 4A–D**). Therefore, ethanol (control), palmitic acid, and elaidic acid were added to RW264.7 cells, a murine macrophage cell line, followed by assessment of the secreted cytokines using multicolor flow cytometric analysis. *IL-1β*- or *IL-12*-positive cells were significantly higher (p<0.001) in the groups treated with palmitic acid or elaidic acid, than in the control group. *IL-1β*-positive cells were not significantly different (p=0.850) between the palmitic acid- and elaidic acid-treated groups, while *IL-12*-positive cells were significantly higher (p<0.001) in the elaidic acid-treated group, than in the palmitic acid-treated group (**Figures 4E, F**).

HTHSD Diet Induces an Increase in the Abundance of the Phylum Proteobacteria

Finally, relative abundance of the gut microbiota was investigated using 16s RNA sequencing. PCoA plots were constructed to compare the three groups (**Figure 5A**). Moreover, beta-diversity with PERMANOVA tests were shown

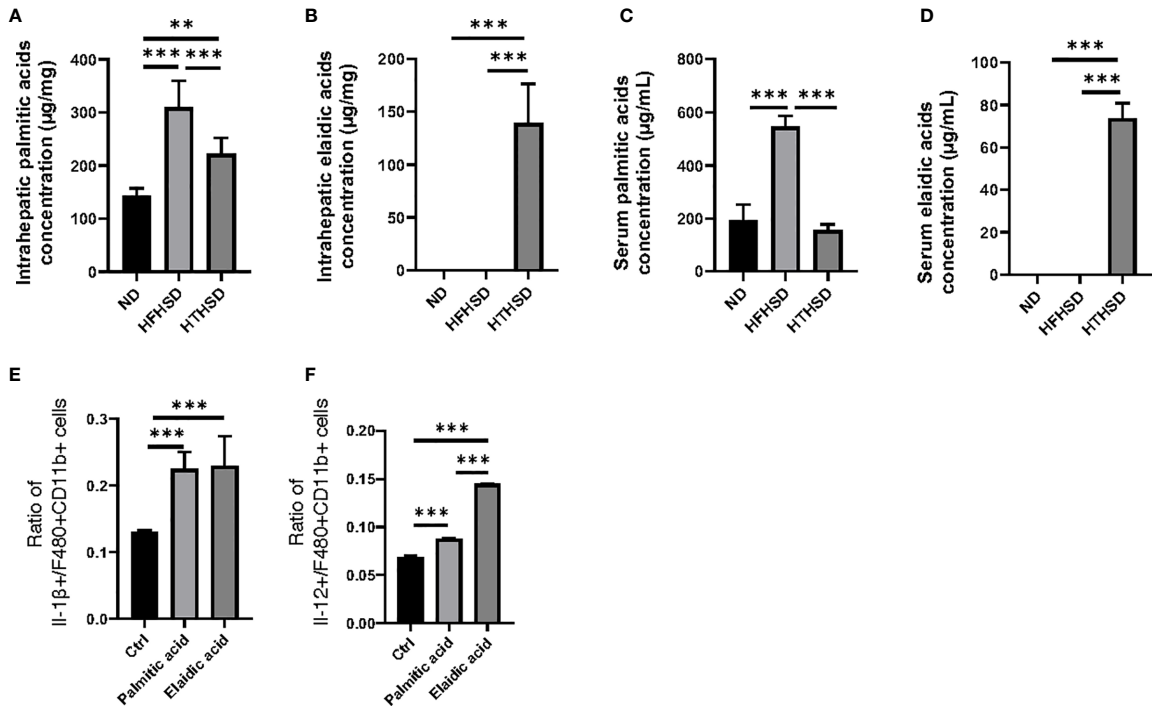


FIGURE 4 | The effect of elaidic acid on IL-1β secretion from macrophages was comparable to that of palmitic acid, and the effect of elaidic acid on IL-12 secretion was significantly stronger. **(A)** Intrahepatic palmitic acid concentration (μg/mg) (n=6). **(B)** Intrahepatic elaidic acid concentration (μg/mg) (n=6). **(C)** Serum palmitic acid concentration (μg/mL) (n=6). **(D)** Serum elaidic acid concentration (μg/mL) (n=6). Data are represented as mean ± SD; **p < 0.01, ***p < 0.001 using one-way ANOVA and an unpaired, two-tailed Student's t test.

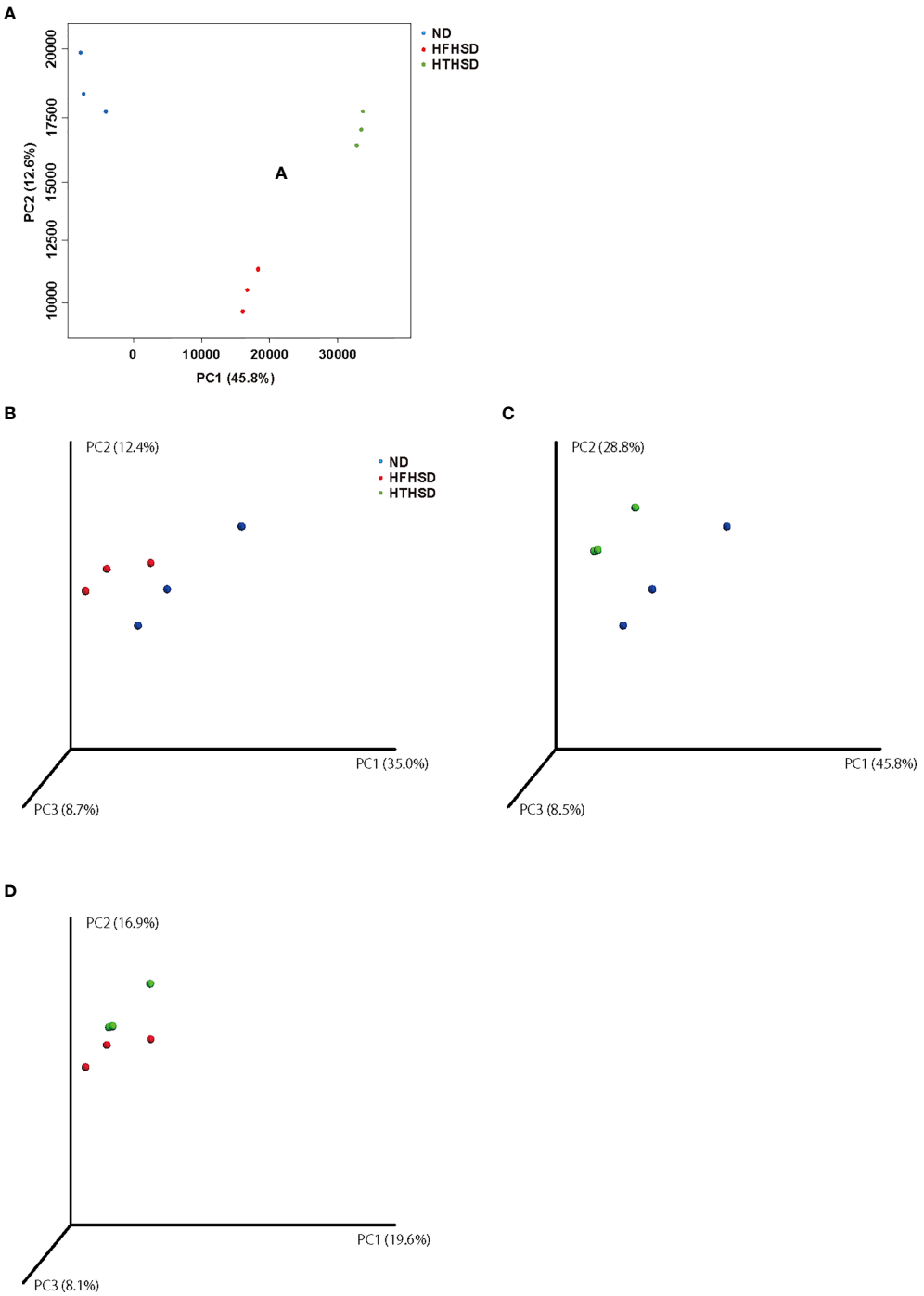


FIGURE 5 | PCoA plots of gut microbiota of mice fed with ND, HFHSD, or HTHSD **(A)** Weighted PCoA plots were shown (n=3). Weighted beta-diversity with PERMANOVA test were shown (n=3). **(B)** ND vs HFHSD (p= 0.010), **(C)** ND vs HTHSD (p= 0.007), and **(D)** HFHSD vs HTHSD (p= 0.055). PCoA: Principal coordinate analysis, PERMANOVA: Permutational multivariat analysis of variance.

in **Figures 5C, D**, and showed notable differences between the ND and HFHSD groups (p= 0.010) or the ND and HTHSD groups (p= 0.007). On the other hand, there was no significant differences between HFHSD and HTHSD groups (p= 0.055) (**Figures 5B–D**). At the phylum level, the relative abundance of *Bacteroidetes* was significantly higher (p<0.001) in mice fed with ND (54.1 ± 14.0%) than in mice fed with HFHSD (21.9 ± 6.8%) or HTHSD (13.5 ± 10.3%); there was no significant difference (p=0.301) between the HFHSD and HTHSD groups (**Figure 6A**). Likewise, the relative abundance of *Deferribacteres* was

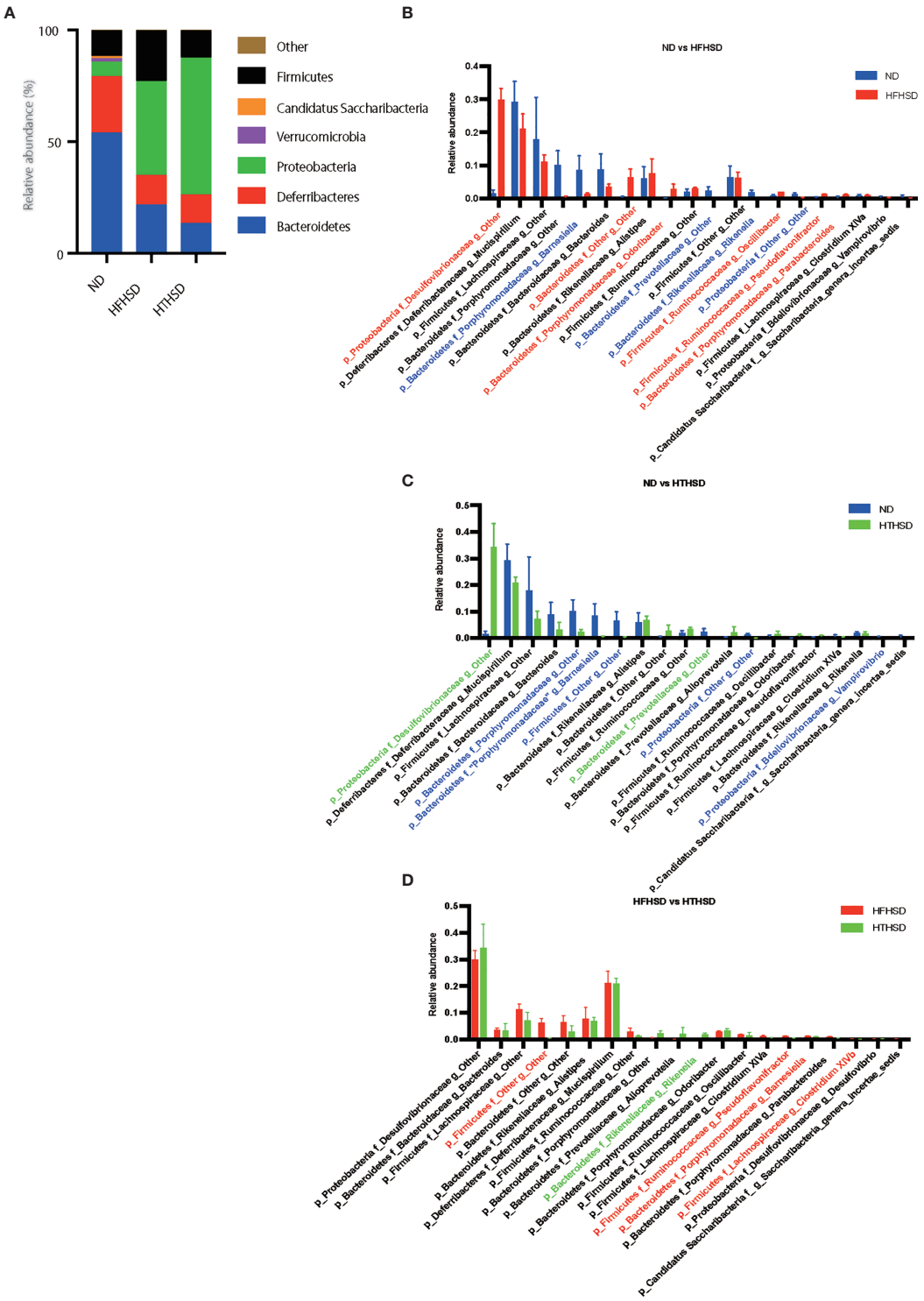


FIGURE 6 | Mice fed with HFHSD or HTHSD displayed significantly higher relative abundance of family *Desulfovibrionaceae* (which belongs to phylum *Proteobacteria)* than mice fed with ND. **(A)** Relative abundance of gut microbiota, by phylum. **(B–D)** Dominant gut microbial genera. The influence of genera of unique gut microbiota in mice fed with ND, HFHSD, or HTHSD was assessed using the weighted average differences method, followed by ranking of the assessed influence of genera from top to bottom. The top 20 gut microbial genera are shown. Relative abundance of gut microbiota in the two groups was compared using an unpaired, two-tailed Student's t test. Gut microbiota with significantly higher abundance in a group have been indicated in the color of the group. *Red*: ND, *Blue*: HFHSD, *Green*: HTHSD.

significantly higher (p<0.001) in mice fed with ND (25.1 ± 5.9%) than in mice fed with HFHSD (13.1 ± 2.8%) or HTHSD (12.9 ± 2.5%); there was no significant difference (p=0.931) between the HFHSD and HTHSD groups. On the contrary, the relative abundance of *Proteobacteria* was significantly higher in mice fed with ND (6.5 ± 2.8%), HFHSD (42.0 ± 3.6%), and HTHSD (61.3 ± 9.9%) (all p<0.001) (**Figure 6A**). At the genus level, the top 20 species were ranked using the WAD method. Upon comparing mice fed with ND and HFHSD, the relative abundance of the family *Desulfovibrionaceae*, which belongs to the phylum *Proteobacteria* (29.7 ± 3.6% vs. 1.4 ± 1.1%, p=0.005), genus *Odoribacte*, genus *Oscillibacte*, genus *Pseudoflavonifractor*, and genus *Parabacteroides* was significantly higher in mice fed with HFHSD, than in mice fed with ND. On the contrary, genus *Barnesiella*, family *Prevotellaceae*, and genus *Rikenella* were significantly lower in mice fed with HFHSD than in those fed with ND (**Figure 6B**). Upon comparing mice fed with ND and HTHSD, the relative abundance of the family *Desulfovibrionaceae* was significantly higher in mice fed with HTHSD than in mice fed with ND (3.3 ± 0.9% vs. 1.9 ± 0.9%, p=0.009). In contrast, the relative abundance of genus *Barnesiella*, family *Prevotellaceae*, and genus *Vampirovibrio* was significantly lower in mice fed with HFHSD, than in mice fed with ND (**Figure 6C**). On the other hand, there were no big differences in the relative abundance of genera between mice fed with HTHSD or HFHSD. However, the relative abundance of genus *Rikenella*, which belongs to the family *Rikenellaceae*, was significantly higher in mice fed with HTHSD, than in mice fed with HFHSD (1.7 ± 0.8% vs. 0.0 ± 0.0%, p=0.018). The relative abundance of the family *Desulfovibrionaceae* was higher in mice fed with HTHSD, than in mice fed with HFHSD, although the difference was not statistically significant. Moreover, the relative abundance of phylum *Firmicutes*, genus *Psudoflavonifractor*, genus *Barnesiella*, and genus *Clostridium XIVb* was significantly lower in mice fed with HTHSD, than in mice fed with HFHSD (**Figure 6D**). Furthermore, in one-way ANOVA, the similar results were obtained (**Supplementary Figure 3**).

DISCUSSION

The present study revealed that, as compared to saturated fatty acids, trans fatty acids induced more intestinal inflammation and resulted in significantly impaired glucose tolerance with increased hepatic fat accumulation and progression of liver fibrosis. Previous studies have shown that trans fatty acids are risk factors for cardiovascular disease, diabetes, and cancer (1, 21, 22). This study provides a basis for this increased risk.

Some possible explanations for this observation are listed below. Secretion of *Il-6*, a pro-inflammatory cytokine, has been found to be higher in mice fed with an elaidic acid-rich diet, as compared to mice fed with a PUFA-rich diet (23). In addition, activation of *NF-κB* and *Tnfa*, as well as expression of *Ccl2*, osteopontin, and macrophage markers in the liver have been found to be elevated in mice fed with a trans fatty acid-rich diet (24–26). Hirata et al. (27) showed that, as compared to both control and oleic acid, 12 h of incubation with 0.2 mM elaidic acid induced caspase 3 cleavage and promoted apoptotic cell death in RAW264.7 macrophages, mediated *via* over-activation of the apoptosis signal-regulated kinase 1-p38 mitogen-activated protein kinase pathway. Ge et al. (9) reported that a diet enriched with trans fatty acids induced lipid deposition in small intestinal epithelial cells and destruction of the small intestinal epithelium. In addition, a significant increase in the expression levels of Cxcl12, Cxcl14, and Cxcr4 has been observed in the small intestine of mice fed with trans fatty acids. In the present study, the number of M1 macrophages and ILC1 in the mucous membranes of the liver and small intestine were predominantly increased in the trans-fatty acid-fed mice, and the expression levels of inflammation-related genes, such as *Tnfa*, *Il6*, and *Il1b*, were also predominantly increased. In addition, the pathological images of small intestine showed that the HFHSD group had a significant decrease in the height and width of the villi and an increase in the depth of the crypt, indicating inflammation of the small intestinal mucosa.

Ge et al. (9) also reported that trans fatty acid-rich diet caused a decrease in the relative abundance of the phylum *Bacteroidetes*, along with an increase in the phylum *Proteobacteria* and the family *Desulfovibrionaceae*, which are gram-negative sulfate-reducing bacteria found to be significantly abundant in obese and metabolically-impaired mice (28, 29). The results of the present study are consistent with those of the above studies. Increased relative abundance of *Desulfovibrionaceae* and the resulting excessive hydrogen sulfide (H_2S) production may contribute to IBDs and inflammation-related bowel diseases, such as colorectal cancer (30). H_2S enhances the breakdown of the mucus barrier by decreasing the disulfide bonds in the mucus network, thus leading to an increase in the permeability of the mucus layer (31). Rupture of the mucus barrier allows toxins and bacteria to come into close proximity to the colonic epithelium, causing inflammation, and ultimately, contributing to the development of IBD and colorectal cancer (31). Furthermore, failure of the mucus barrier has been reported to alter the innate immunity of the intestinal tract. In particular, the number of ILC3s, which are major regulators of inflammation and infection at mucosal barriers, are altered by failure of the mucus barrier (32). ILC3-derived IL-22 plays an important role in promoting STAT3-dependent expression of antimicrobial peptides and maintenance of the intestinal epithelial barrier function (33–35). Conversely, loss of ILC3s is associated with reduced expression of IL-22 and lower levels of antimicrobial peptides expressed by the intestinal epithelial cells. In the present study, intake of HFHSD or HTHSD increased the abundance of the family *Desulfovibrionaceae* in the gut microbiota and decreased the number of ILC3s in the lamina propria of the small intestine. Additionally, the increased abundance of the family *Desulfovibrionaceae* has been reported to be associated with increased fat absorption (36, 37). In our study, expression of Cd36, a transporter of long-chain fatty acids, in the small intestine was significantly higher in mice fed with HFHSD or HTHSD, than in those fed with ND; between the HTHSD and HFHSD groups, HTHSD displayed higher expression. At the same time, mice fed with HTHSD displayed

higher abundance of the family *Desulfovibrionaceae*, as compared to those fed with HFHSD, although the difference was not significant. Moreover, the relative abundance of the genus *Rikenella*, which belongs to the family *Rikenellaceae*, was significantly higher in mice fed with HTHSD than in those fed with HFHSD. Several previous studies have reported that the abundance of the family *Rikenellaceae* increases upon intake of a high-fat diet (38), and this increase is related to the loss of gut barrier function (39).

In summary, the failure of the mucus barrier was more severe in the mice fed with HTHSD, than in the mice fed with HFHSD, due to an increase in the relative abundance of the family *Desulfovibrionaceae* and genus *Rikenella*, which might be responsible for causing various metabolic disorders.

The increased ratio of ILC1s and decreased ratio of RORγt-positive ILC3s in the small intestine were observed to a similar degree in both mice fed with HFHSD and HTHSD, whereas the ratio of T-bet-positive ILC3s in the small intestine was significantly higher in mice fed with HTHSD, than in mice fed with HFHSD. There is some evidence that ILCs can exhibit functional plasticity in response to environmental cues. The function of ILC3s has been shown to vary with the expression of the transcription factors RORγt and T-bet (40). Stimulation of cytokines such as IL-12 and IL-18 increases the number of ex-RORγt-positive ILC3s, which are characterized to be T-bet positive, and decreases the number of RORγt-positive ILC3s, indicating that ILC3s are able to respond to environmental cues. It has been reported that ex-RORγt-positive ILC3s have the ability to produce IFNγ and reduce the production of IL-17 and IL-22 (41). Thus, ex-RORγt-positive ILC3s exhibit functions similar to those of ILC1s. In addition, in cell experiments using RAW264.7, elaidic acid, which was elevated in the liver and serum samples of mice fed with HTHSD, did not significantly increase the ratio of IL-1β-positive cells, as compared to palmitic acid, which was elevated in the liver and serum samples of mice fed with HFHSD. IL-1β has been reported to accelerate the dedifferentiation of ILC1s to ILC3s (42). It has been suggested that there is a compensatory increase in IL-1β secretion, to increase the number of ILC3s, which attenuates intestinal mucosal inflammation. On the other hand, as compared to cells administered with palmitic acid, the ratio of IL-12-positive cells was significantly higher in cells administered with elaidic acid. Therefore, it is thought that, as compared to palmitic acid stimulation, IL-12 secreted by elaidic acid stimulation of macrophages increases the number of ILC1s (43) and differentiates ILC3s into ex-RORγt-positive ILC3s to a greater extent. In this study, intake of HTHSD might have decreased the production of IL22s in intestinal epithelial tissues by increasing the number of ex-RORγt-positive ILC3s, which strongly express T-bet and lack the ability to produce IL-22, thus reducing the maintenance of antimicrobial peptides and intestinal epithelial barrier function. On the other hand, the number of ILC3s in the liver was significantly higher in the HFHSD or HTHSD groups, than in the ND group, a trend opposite to that observed in the case of ILC3s in the lamina propria of the small intestine. In our previous study, we found that HFD treatment caused a compensatory increase in the number of ILC3s, to reduce inflammation in the liver (11). In the present study, inflammation in the small intestinal mucosa was the primary and important change, while an increase in the number of ILC3s in the liver was thought to be compensatory, due to the associated hepatitis and liver fibrosis.

As limitation of this study, we do not have the data of the microbiome longitudinally i.e., at baseline and at several timepoint. This data could have clarified the effect of HFSHSD and HTHSD in changing gut microbiota.

Taken together, the present study revealed that the intake of trans-fatty acids and saturated fatty acids caused dysbiosis and associated immune changes in the intestine, and significantly aggravated metabolic diseases such as diabetes and fatty live when compared with the intake of normal diet, and this was more pronounced for trans-fatty acids. In addition, trans-fatty acids strongly activate the differentiation of RORgt-positive ILC3 into T-bet-positive ILC3 by promoting the secretion of IL-12 from macrophages more strongly than saturated fatty acids.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://doi.org/10.6084/m9.figshare.14370035.v1.

ETHICS STATEMENT

The animal study was reviewed and approved by The Committee for Animal Research at the Kyoto Prefectural University of Medicine.

AUTHOR CONTRIBUTIONS

TO originated and designed the study, researched the data, and wrote the manuscript. YH and MH originated and designed the study, researched the data, and reviewed the manuscript. SM, TS, EU, NN, MA, and MY researched the data and contributed to the discussion. HT provided technical cooperation. MF originated and designed the study, researched the data, and reviewed and edited the manuscript. MF is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors contributed to the article and approved the submitted version.

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Gut Microbiota Dysbiosis Induced by Intracerebral Hemorrhage Aggravates Neuroinflammation in Mice

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Intracerebral hemorrhage (ICH) induces a strong hematoma-related neuroinflammatory reaction and alters peripheral immune homeostasis. Recent research has found that gut microbiota plays a role in neurodegeneration and autoimmune diseases by regulating immune homeostasis and neuroinflammation. Therefore, we investigated the relationship between ICH, microbiota alteration, and immune responses after hematoma-induced acute brain injury. In our study, we used a mouse model of ICH, and 16S ribosomal RNA sequencing showed that ICH causes gut microbiota dysbiosis, which in turn affects ICH outcome through immune-mediated mechanisms. There was prominent reduced species diversity and microbiota overgrowth in the dysbiosis induced by ICH, which may reduce intestinal motility and increase gut permeability. In addition, recolonizing ICH mice with a normal health microbiota ameliorates functional deficits and neuroinflammation after ICH. Meanwhile, cell-tracking studies have demonstrated the migration of intestinal lymphocytes to the brain after ICH. In addition, therapeutic transplantation of fecal microbiota improves intestinal barrier damage. These results support the conclusion that the gut microbiome is a target of ICH-induced systemic alteration and is considered to have a substantial impact on ICH outcome.

Keywords: intracerebral hemorrhage, neuroinflammation, gut microbiota, dysbiosis, T cell

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INTRODUCTION

Hemorrhagic stroke is a common type of stroke with a poor prognosis and high mortality rate (Chang et al., 2018; Zhu et al., 2019). Brain injuries after intracerebral hemorrhage (ICH) involve the following: primary injury induced by hemorrhage and hematoma growth and secondary injury caused by a series of pathologic responses (Zhao et al., 2014; Duan et al., 2016; Zhu et al., 2019). Previous studies have shown that surgical hematoma evacuation may reduce mass effects and hematoma-related brain injury (Wilkinson et al., 2018). However, to date, this method has failed to improve long-term functional outcomes in ICH patients (Sattur and Spiotta, 2020). Therefore, a novel therapeutic target for ICH is imperative.

Previous basic and clinical research has indicated that neuroinflammation contributes to the progression of ICH-induced brain injury (Zhu et al., 2019). Neuroinflammation after ICH is a

complex process that is mediated by cellular and molecular components (Wang and Dore, 2007). Previous studies have shown that T cells play a defining role in secondary neuroinflammation after acute brain injury (Tschoe et al., 2020). T cell function has been well characterized in ischemic stroke, but its role in ICH is poorly defined (Shichita et al., 2012). Nonetheless, some studies have shown that pro-inflammatory T cells promote vascular permeability and exacerbate brain injury after ICH *via* the production of inflammatory cytokines (Arumugam et al., 2005). In addition, T cells can communicate with microglia and promote microglial polarization into the M1 phenotype (pro-inflammation), which can exacerbate ICH-induced neuroinflammation (Biswas and Mantovani, 2010). Based on the research mentioned earlier, we assumed that T cells might be a potential target for the treatment of ICH.

Evidence suggests that the gut microbiota is a key regulator of T cell homeostasis and neuroinflammation in nervous system diseases. Recently, rapid development in metagenomics, metatranscriptomics, and meta-proteogenomics has revealed the functional relationship between the gut microbiota and the central nervous system (CNS) function, termed the "gut-brain axis," which has become an emerging field in neuroscience and neuroimmunology (Berer et al., 2011; Cryan and Dinan, 2012). Gut microbiota play a decisive role in many CNS diseases, including Alzheimer's disease, Parkinson's disease, and ischemic stroke. A recent study showed that the regulation of gut microbiota influences neuroinflammation and outcome in an experimental stroke model (Singh et al., 2016). However, the impact of ICH on the gut microbiota composition and the contribution of brain injury-specific microbiota alterations on neuroinflammation and outcome after brain injury are still unknown. Therefore, this study aimed to investigate gut microbiota alterations after ICH and its role in the regulation of neuroinflammation reaction induced by ICH.

In our study, we found that gut microbiota dysbiosis is induced by ICH. In turn, gut microbiota instability causes changes in T cell homeostasis, induction of pro-inflammatory response, and deterioration of outcome. Fecal microbiota transplantation (FMT) to normalize post-hemorrhage dysbiosis is associated with an improved neurobehavioral function and ameliorated neuroinflammation. As mentioned earlier, our results point to a novel and highly complex interplay between the brain and the gut microbiota after acute brain injury, in which the gut microbiota are a target of ICH-mediated pathways resulting in dysbiosis as well as an effector of immune homeostasis with profound impact on ICH outcome.

MATERIALS AND METHODS

Animals

Adult male C57 mice (10–12 weeks old, 22–25 g) of specific pathogen-free grades were purchased from Vital River Laboratory Animal Co., Ltd. (Beijing, China). These mice were provided *ad libitum* with access to food and water and housed under a 12/12-h dark/light cycle in specific pathogen-free conditions (three mice per cage). The mice were randomly assigned to the sham and experimental groups after adaptive feeding. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Institutional Ethics Committee of the Second Affiliated Hospital, Zhejiang University of Medicine.

Study Design

All subjects in this study were blinded to the treatment during the experiments and outcome assessment.

Experiment 1

To investigate changes in the gut microbiome after ICH, ICH was induced in five mice by injecting collagenase into the basic ganglia region. Fecal matter was collected pre-ICH and ICH (3 days), then the 16S ribosomal RNA (rRNA) gene sequencing was performed.

Experiment 2

Thirty-six mice were randomly assigned into the following two groups to investigate the impaired function of the gastrointestinal trace after ICH: sham (n = 15), ICH 3 days (n = 15), ICH 7 days (n = 3), and ICH 14 days (n = 3). The complete gastrointestinal tract was removed from the mice (six per group) to assess gastrointestinal motility. Gut permeability was analyzed by plasma fluorescence measurements (n = 6). Change in gut micro-construction after ICH was assessed by hematoxylin and eosin staining (per group n = 3).

Experiment 3

Twelve mice were randomly divided into three groups to study the temporal dynamics of T cells in the brain after ICH: sham (n = 3), ICH 1 day (n = 3), ICH 3 days (n = 3), ICH 7 days (n = 3), and ICH 14 days (n = 3). Hemorrhage hemispheres were collected for immunofluorescence and flow cytometry. The corresponding antibodies were used to label and identify the different T cells.

Experiment 4

Twelve mice were randomly divided into two groups to study the migration of leukocytes after ICH: sham (n = 6) and ICH 3 days (n = 6). Peyer's patches (PPs) labeled by fluorescent cell staining dyes carboxyfluorescein succinimidyl ester (CFSE) were used for flow cytometry (n = 3 per group), whereas those labeled with CM-Dil were used for immunofluorescence (n = 3 per group).

Experiment 5

To investigate the changes in neuroinflammation after FMT in ICH mice, 18 mice were randomly divided into five groups: sham (n = 6), vehicle 7 days (n = 3), vehicle 14 days (n = 3), ICH + FMT 7 days (n = 3), and ICH + FMT 14 days (n = 3). The ipsilateral basal ganglia region samples of the mice were collected for quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

ICH + FMT group mice were treated with the fecal supernatant (100 μl/day, by gavage) from healthy mice, whereas the vehicle group mice were treated with vehicle [100 μl of phosphate-buffered saline (PBS)/day, by gavage] during the same time.

Experiment 6

To investigate the effect of microbiota on neurobehavioral function after ICH, healthy microbiota were transplanted into ICH mice. Sixteen mice were randomly divided into two groups (vehicle and ICH + FMT group) for the subsequent experiments. Bodyweight and neurological scores were evaluated in each group at 3, 7, 14, and 28 days after ICH ($n = 8$).

Experiment 7

To investigate the causal relationship between gastrointestinal motility and dysbiosis in the gut microbiota, 12 mice were randomly divided into two groups: sham ($n = 6$) and surgical ileus ($n = 6$). The complete gastrointestinal tract was removed from the mice (three per group) to assess their gastrointestinal motility. Three days after surgery, their fecal matters were collected, and 16S rRNA gene sequencing was performed and compared with the composition of their fecal matter presurgery.

Experiment 8

To investigate changes in the gut barrier after FMT in ICH mice, 21 mice were randomly divided into three groups: sham ($n = 7$), vehicle 7 days ($n = 7$), and ICH + FMT 7 days ($n = 7$). Colon samples from the mice were collected for immunofluorescence analysis ($n = 3$ per group). Gut permeability was analyzed by plasma fluorescence measurements ($n = 4$).

Intracerebral Hemorrhage Mouse Model

The ICH mouse model was performed as described previously (Taylor et al., 2017; Chang et al., 2018). Briefly, mice were anesthetized with pentobarbital sodium (40 mg/kg, 1%) *via* intraperitoneal injection. The 0.05 U type VII collagenase (Clostrid-iumhistolyticum; Sigma-Aldrich) prepared in 0.5-μl saline was stereotactically injected into the right basal ganglia (2.5-mm lateral to the bregma, 3-mm deep at a 5° angle). Throughout the surgery, the rectal temperature was maintained at 37.0 ± 0.5°C. The sham mice received the same treatment, including needle insertion, but without collagenase injection.

Gut Microbiota Analysis

16S rRNA amplicon sequencing was performed by LC-Bio Technology Company (Hangzhou, Zhejiang, China) to analyze gut microbiota. Fresh feces were collected by abdominal massage to avoid contamination by exogenous bacteria. DNA from mouse feces was isolated by using an E.Z.N.A. ®Stool DNA Kit (D4015, Omega, Inc., United States) according to the manufacturer's instructions. The total amount of DNA in each sample was measured using a Qubit fluorometer (Thermo Fisher, MA, United States). PCR amplification of the 16S rRNA sequence was performed using primer sets specific to V4 regions. Final PCR products were purified using AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, United States) and quantified using Qubit (Invitrogen, United States). The amplicon pools were prepared for sequencing, and the size and quantity of the amplicon library were assessed on an Agilent 2100 Bioanalyzer (Agilent, United States) and with the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, United States), respectively. The libraries were sequenced using the NovaSeq PE250 platform.

Samples were sequenced on an Illumina NovaSeq platform according to the manufacturer's recommendations provided by LC-Bio. Paired-end reads were assigned to samples based on their unique barcodes and truncated by cutting off the barcode and primer sequences. Paired-end reads were merged using the FLASH software. Quality filtering of the raw reads was performed under specific filtering conditions to obtain the high-quality clean tags according to the formula (v0.94). Chimeric sequences were filtered using Vsearch software (v2.3.4). After dereplication using DADA2, we obtained a feature table and feature sequence. Alpha diversity and beta diversity were calculated by normalizing the same sequences randomly. Then, according to the SILVA (release 132) classifier, the feature abundance was normalized using the relative abundance of each sample. Alpha diversity was applied to analyzing the complexity of species diversity for a sample through five indices, including Chao 1, Observed species, Goods coverage, Shannon, and Simpson, and all these indices in our samples were calculated with QIIME2. Beta diversity was calculated using QIIME2, and graphs were drawn using the R package. BLAST was used for sequence alignment, and the feature sequences were annotated with the SILVA database for each representative sequence. Other diagrams were implemented using the R package (V3.5.2).

Gastrointestinal Motility Analysis

The mice received 100 μl of fluorescein isothiocyanate-dextran 70 [70,000 Da fluorescein isothiocyanate (FITC)-dextran, 50 mg/ml, Sigma] in 0.9% saline. One hour after administration, the mice were killed, and the entire intestinal tract from the stomach to the colon was removed, and images were acquired using a chemiluminescence detection system (IVIS spectrum, Perkin Elmer). To quantify gastrointestinal motility, the complete gastrointestinal tract was divided into different segments. Each segment was chopped, the liberated luminal contents were homogenized for 1 min, tissue and coarse particles were removed by centrifugation (300 × *g*, 5 min), and the fluorescence of the supernatant was measured using a fluorescence spectrophotometer (SoftMax®Pro5, Molecular Devices). The value obtained was normalized to that of the blank controls and expressed as the percentage of fluorescence per intestinal segment.

Intestinal Permeability Assay in Mice

In vivo intestinal permeability was assessed by oral gavage of the fluorescein isothiocyanate-dextran 4 (600 mg/kg, 100 mg/ml; 4000 Da FITC-Dextran, Sigma) in mice that had been fasted for 6 h. Blood was collected 2 h after gavage, and plasma was prepared by centrifugation at 2,500 × *g* for 10 min. The fluorescence intensity of undiluted plasma was analyzed using a fluorescence spectrophotometer (SoftMax®Pro5, Molecular Devices) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The value obtained was normalized to that of the blank controls and expressed as the percentage of fluorescence per mouse. During this process, the researcher was blinded to the experiment.

Histopathology of Ileum and Colon

Excised ileum and colon tissues were fixed in 4% paraformaldehyde (PFA) and embedded in paraffin. The blocks were serially cut into 5-μm thick sections and stained with hematoxylin and eosin staining. Histological images were obtained using a microscope (Leica, Mannheim, Germany).

Tracing Migration of Leukocytes From Peyer's Patches to the Brain

Twenty-four hours after ICH and sham surgery, cells within PPs were labeled by microinjection of fluorescent cell staining dyes CFSE (25 μM in 2 μl of PBS per PP) or CM-Dil (5 μM in 2 μl of PBS per PP) (Invitrogen) as previously described (Singh et al., 2016). Mice were killed 48 h after cell labeling, and brains were prepared either for flow cytometric analysis (CFSE labeling) or immunofluorescence (CM-Dil labeling).

Fecal Microbiota Transfer

Fresh fecal pellets were collected from five healthy mice between 9 and 10 am and diluted in ice-cold PBS (120 mg feces/1-ml PBS). Briefly, the stool was steeped in cold PBS for approximately 5 min, homogenized for 10 min, and then centrifuged at 1,000 × *g* at 4° for 10 min. The supernatant was transferred to new tubes and used for transplantation. Recipient mice were orally gavaged with streptomycin (500 mg/ml) in 50-μl sterile PBS for the first 2 days after the ICH. The premise of the antibiotic treatment was to decrease the bacterial load in the recipient mice to reduce competition for the repopulation of microbiota from the donor mice. Three days after ICH, 100 μl of fecal supernatant was gavaged to recipient mice daily for 7 days.

Assessment of Neurobehavioral Function

Behavioral function assessments were performed by two researchers who were blinded to the experiment. Three tests were used to evaluate neurobehavioral function from 3 to 28 days after ICH as previously described (Hua et al., 2002). For the forelimb placing test, each mouse was held by its torso, allowing the forelimb to hang free. Each forelimb was tested 10 times per mouse, and the percentage of trials in which the mouse placed the appropriate forelimb on the edge of the countertop in response to the vibrissae stimulation was determined. For the cylinder test, mice were placed in a transparent cylinder (diameter: 8 cm; height: 25 cm) and allowed to rear 20 times freely. The location of the first forelimb on the wall was recorded. A score was calculated as follows: (right - left)/(right + left + both), in which a greater positive score indicated more severe left hemiparesis. For the corner turn test, the mice could proceed into a 30° corner and then freely turn either right or left to exit the corner. The choice of direction during 10 trials was recorded, and the percentage of right turns was calculated.

Quantitative Real-Time Polymerase Chain Reaction

Mice were anesthetized, intracardiac perfusion was performed with 0.1 mol/L cold PBS, and the brain was gently collected and trimmed. Total RNA from the hematoma basal ganglia region was isolated using TRIzol reagent (Invitrogen, Thermo Fisher, MA, United States), according to the manufacturer's protocol. Complementary DNA was synthesized using a PrimeScript™ RT Master Mix (Takara Bio Inc, Shiga, Japan). qRT-PCR was performed using a standard protocol, with Applied Biosystems Quant Studio™ 5 (Thermo Fisher Scientific, Waltham, MA, United States) and TB Green™ Premix Ex Taq™ (Takara BioInc, Shiga, Japan). The specificity of each reaction was evaluated using melting curve analysis. β-actin was used as an internal control. Each reaction was performed in triplicate, and the change in relative target gene expression normalized to the internal control levels was determined using the $2^{-\Delta\Delta Ct}$ method (Donato et al., 2020). The sequences of the gene-specific primers (Sangon Biotech, Shanghai, China) are listed in **Table 1**.

Immunofluorescence

As previously described, mice were deeply anesthetized and perfused with 20-ml ice-cold 0.1 mol/l PBS, followed by 4% PFA. The brain was collected and then fixed with 4% PFA overnight and 30% sucrose for 72h at 4°C. Then, the brain samples were cut into 10-μm coronal slices for subsequent experiments. The colon tissues were fixed in 4% PFA overnight and cut into 5-μm thick sections. The tissue sections were washed with PBS and incubated with 10% donkey serum containing 0.3% Triton X-100 for 1 h at room temperature. The sections were then incubated overnight with the following antibodies: CD3 (1:50, Santa Cruz Biotechnology, sc-18843), Occludin (1:50, Santa Cruz Biotechnology, sc-133256), and Claudin (1:50, Santa Cruz Biotechnology, sc-166338) at 4°. Then, the sections were washed with PBS and incubated with Alexa Fluor 488-conjugated donkey anti-Rat immunoglobulin G (IgG) (Invitrogen, A-21208), Alexa Fluor 488-conjugated donkey anti-mouse IgG (Invitrogen, A-21202), and Alexa Fluor Plus 594-conjugated donkey anti-mouse IgG (Invitrogen, A-32744) at 37° for 1 h. Finally, the sections were stained with 4′,6-diamidino-2-phenylindole (Abcam, ab104135) and observed under a fluorescence microscope (Leica, Mannheim, Germany). Three sections were examined per mouse. Each brain section was examined under three fields of vision to acquire the mean number of target cells, and each colon section was examined for mean fluorescence intensity using Image J software (NIH).

TABLE 1 | Primers used in RT-PCR.

	Primer sequences (5′-3′)	
Gene	**Forward**	**Reverse**
IL-17	CCCCTTCACTTTCAGGGTCG	CCCCTTCACTTTCAGGGTCG
Foxp3	AGTCTGCAAGTGGCCTGGTT	TGCTCCAGAGACTGCACCAC
IFN-γ	CTGGAGGAACTGGCAAAAGGATGG	GACGCTTATGTTGTTGCTGATGGC
IL-β	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA
iNOS	CAAGCACCTTGGAAGAGGAG	AAGGCCAAACACAGCATACC
TNF-α	ATGGCCTCCCTCTCAGTTC	TTGGTGGTTTGCTACGACGTG
β-Actin	AGGCATTGTGATGGACTCCG	AGCTCAGTAACAGTCCGCCTA

iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor-alpha.

Flow Cytometry

Single-cell suspensions were prepared as described previously (Garcia-Bonilla et al., 2015; Posel et al., 2016). Briefly, mice were anesthetized and perfused with 20 ml of ice-cold 0.9% saline to eliminate blood cells. The brain was dissected, minced with fine scissors, and enzymatically digested in Hanks' balanced salt solution with Liberase Dispase High (62.5 μg/ml, Roche Diagnostics) and DNase I (50 U/ml, Beyotime, Shanghai, China) for 1 h at 37° with gentle agitation. After the digestion, the tissue samples were triturated and passed through a cell strainer (70 μm). The cells were washed and subjected to 5 ml of 25% Percoll density gradient centrifugation (25 min, 800 × *g*, 4o). The myelin coat and the supernatant were carefully aspirated, and the cell pellet was preserved at the bottom. The cells were resuspended in 3% fetal bovine serum prepared in a fluorescence-activated cell sorting buffer. After that, the cells were incubated with a mixture of antibodies at 4° for 20 min in the dark for cytometric analysis. The antibodies used in the present study included the following: CD45-Alexa Flour 700 (1:100, eBioscience™, 56-0454-82), CD3-PE (1:100, eBioscience™, 12-0032-82), CD4-APC (1:200, eBioscience™, 17-0042-82), CD8-eFlour450 (1:100, eBioscience™, 48-0081-82), and CD25-Alexa Flour 488 (1:100, eBioscience™, 53-0251-82). For intracellular staining, cells were fixed and permeabilized using Foxp3/Transcription Factor Staining Buffer Set (eBioscience™, 00-5523-00) and stained with Foxp3-PE-Cyanine5 (1:100, eBioscience™, 15-5773-82). After staining was completed, the single cells were analyzed on a CytoFLEX flow cytometer (Beckman Coulter, United States), and the results were analyzed using FlowJo version-10.

Surgical Ileus Mouse Model

A surgical ileus mouse model was used as previously described (Vilz et al., 2012). Briefly, mice were anesthetized with pentobarbital sodium (40 mg/kg, 1%) *via* intraperitoneal injection. The mice were then fixed on a feedback-regulated heating pad with adhesive tape. After shaving and surgical disinfection, the abdominal cavity was then opened 2 cm in length along the median. Two moist sterile cotton applicators were used to place the small intestine content from the pylorus to the cecum. After surgery, the mouse incision was sutured, and mice were placed on a heating lamp until they recovered from anesthesia.

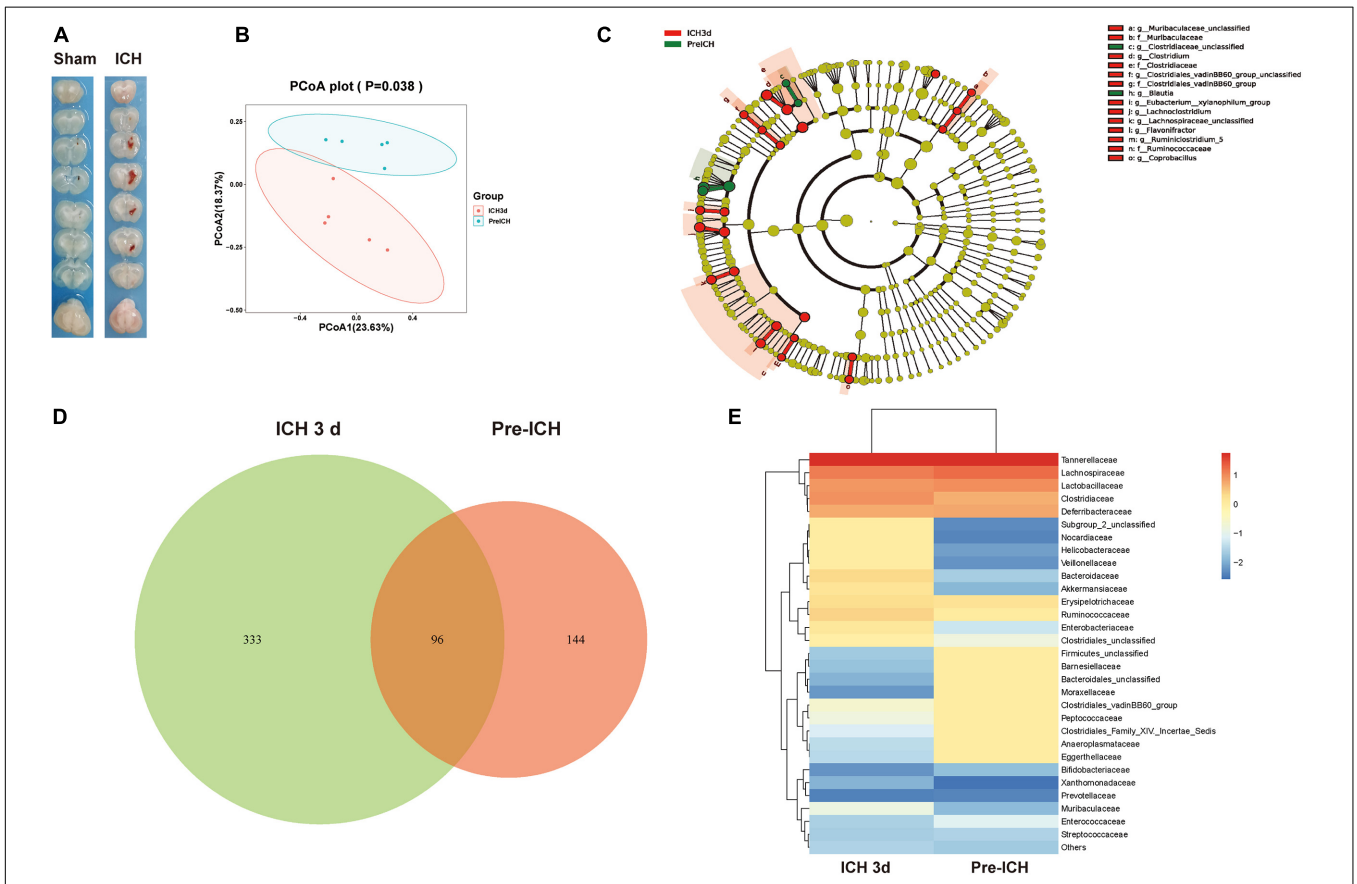

FIGURE 1 | Gut microbiota alterations after intracerebral hemorrhage in mice. **(A)** Images represented of sham and ICH coronal brain sections 3 days after ICH. **(B)** Principal coordinates analysis of the gut microbiota by taxonomic abundance patterns in sham and ICH after 3 days mice (*n* = 5 per group). **(C)** Linear discriminant effect size analysis was performed for indicator taxa analysis identifying features that are statistically different between sham and ICH mice (*n* = 5 per group). **(D)** Venn diagram of the number of differentially features in sham and ICH mice after 3 days (*n* = 5 per group). **(E)** Heat maps of the cluster analysis showing the bacterial composition at the family level in sham and ICH mice after 3 days (*n* = 5 per group).

Statistical Analysis

All data are presented as mean ± standard error of the mean. Data exhibiting the normal distribution and homogeneity of variance between the two groups were compared using the t-test. Persistent neurological functions were analyzed by two-way repeated-measures ANOVA followed by Tukey's *post hoc* test. Data with non-normal distribution and unequal variance were compared using the Kruskal–Wallis test and a Dunn–Bonferroni test for *post hoc* comparisons. Statistical significance was set at $P < 0.05$. Statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Prism Software Inc, San Diego, CA, United States) and SPSS 22.0 for Windows (IBM Corp., Armonk, NY, United States).

RESULTS

Intracerebral Hemorrhage Induces Gut Microbiota Dysbiosis

To investigate whether the ICH is associated with alterations in the intestinal microbiota, we studied the composition of the gut microbiota after ICH in mice using 16S rRNA sequencing of the gut microbiota composition in mice after ICH, with the sham groups showing substantial changes 3 days after ICH (**Figures 1A,B**). Linear discriminant effect size analysis revealed that the composition of the intestinal microbiota between sham and ICH mice was significantly different, as shown in the cladogram (**Figure 1C**). The number of different features between sham and ICH 3 days mice was significantly different (**Figure 1D**). In addition, we have also observed that members of *Firmicutes*, *Barnesiellaceae*, *Bacteriidales*, and *Moraxellaceae* were significantly reduced, whereas *Nocardiaceae*, *Helicobacteraceae*, *Veillonellaceae*, *Bacteroidaceae*, and *Akkermansiaceae* were increased after ICH (**Figure 1E**).

Intracerebral Hemorrhage Induced Brain Injury Impaired Gastrointestinal Function and Increased Gut Permeability

Next, we investigated the mechanisms linking ICH-induced brain injury and dysbiosis of gut microbiota. It has been previously reported that patients with severe brain injury had reduced gastrointestinal motility (Cheng et al., 2018). Consistent with these clinical observations, we detected severe gastrointestinal paralysis after ICH in mice using a gastric fluorescent bolus tracking technique (**Figures 2A,B**). Furthermore, we found that the intestinal barrier integrity at post-ICH day 3 was increased in treated mice compared with the sham group, as reflected by the increased efflux of circulating FITC-dextran (**Figure 2C**). Consistently, ICH also causes histological changes characterized

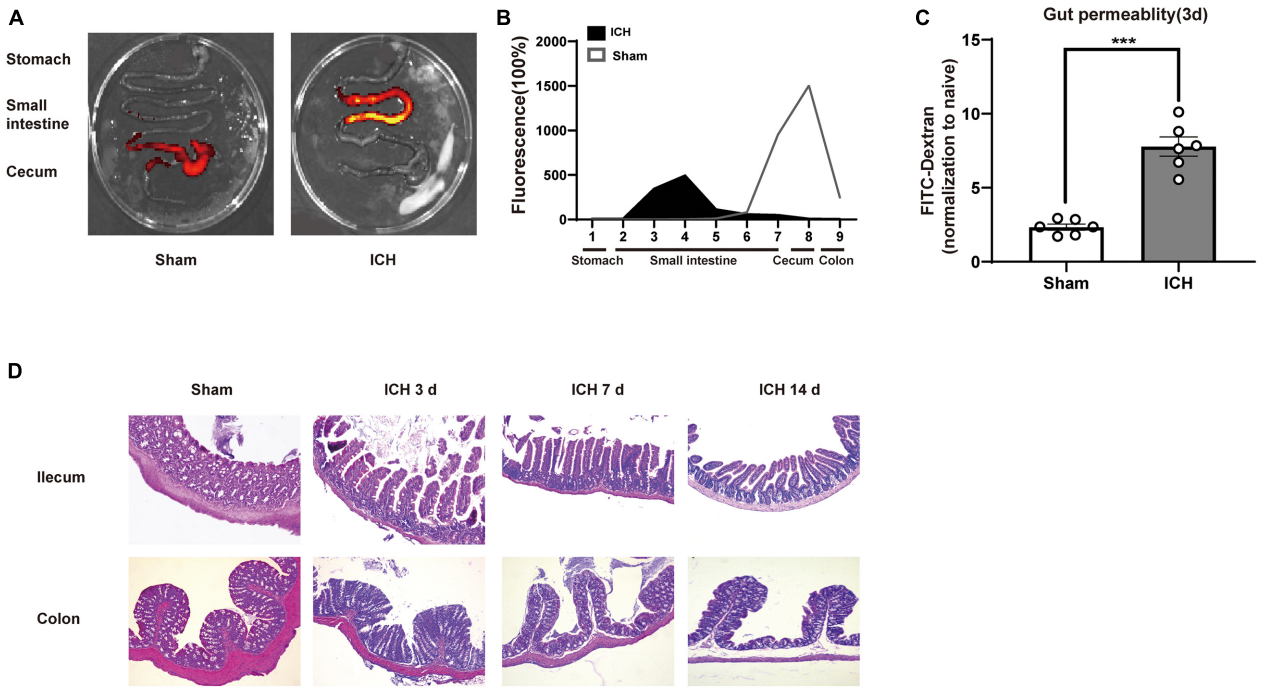

FIGURE 2 | Gastrointestinal dysfunction after ICH. **(A)** Intestinal motility was measured 3 days after sham and ICH surgery. Representative fluorescence images of the complete gastrointestinal tract 1 h after gastric instillation of FITC-dextran showing retention of the fluorescence intensity in intensity segments after ICH. **(B)** Quantification of fluorescent intensity in intestinal segments ($n = 6$ per group). **(C)** Intestinal permeability was measured by oral gavage of FITC-dextran (4 KD) with subsequent analysis of the level plasma of FITC-dextran. There was a significant increase in the ICH mice than the sham group after 3 days. ($n = 6$ per group). **(D)** Intestinal histopathologic changes after ICH 3, 7, and 14 days of ICH. Hematoxylin and eosin staining shows thinning of the layers of epithelium and muscularis mucosae; loss of crypts and glands; edema of the lamina propria; and thickened and shortened villi at the histomorphometric level ($n = 3$ per group). Data are expressed as the mean ± standard error of the mean. ***$P < 0.001$ *vs*. sham group.

by a thinner layer of epithelium and muscularis mucosae, loss of crypts and glands, edema of the lamina propria, and thickened and shortened villi at the histomorphometric level after ICH 3, 7, and 14 days compared with sham mice (**Figure 2D**).

Change in T Cells Type After Intracerebral Hemorrhage

To determine changes in T cell type after ICH, we first used immunofluorescence to examine whether there was T cell infiltration into the perihematomal region. At 7 days after ICH, we found $CD3^+$ cell accumulation around in the perihematomal region (**Figure 3A**). Then, we used flow cytometry to analyze the different types of T cell changes after ICH. At 1, 3, and 14 days, the experiments were performed, and compared with the sham group mice, the $CD4^+$ T cells, $CD8^+$ T cells, and regulatory T lymphocytes (Tregs) were increased in the hemorrhagic hemisphere. The trend of the increase was more significant at 14 days after ICH (**Figures 3B–E**).

The gating strategy for the determination of different T cells is as follows: $CD45^+$ cells were represented the leukocytes, the $CD3^+$ $CD4^+$ population indicates the $CD4^+$ T lymphocytes, the $CD3^+$ $CD8^+$ population represents the $CD8^+$ T lymphocytes, and the $CD25^+Foxp3^+$ population is considered a Tregs population.

T Cells Migrate From the Intestine to the Brain After Intracerebral Hemorrhage

After ICH, the infiltration of T cells in the perihematomal region plays a key role in ICH-induced neuroinflammation. To investigate potential T cell migration from PPs to the brain, we have used a fluorescent labeling technique by microinjection of CFSE and CM-DiI in all detectable PPs of the mouse intestines after ICH and sham surgery (**Figure 4A**). Consistent with a previous report, T cells and monocytes fluorescently labeled after microinjection in PPs were detected in the perihematoma region 3 days after ICH, consistent with the previously demonstrated kinetics of post-ICH leukocyte invasion. CFSE-positive cells derived from labeled PPs were increased after ICH (**Figures 4B,C**). We have also confirmed these findings in an independent experiment using CM-DiI as a lipophilic labeling dye and subsequent histological analysis. Here, superimposing localization of T cells from four mice on one coronal brain section, we have detected brain-invading T cells around the hematoma as previously reported (**Figure 4D**). These results

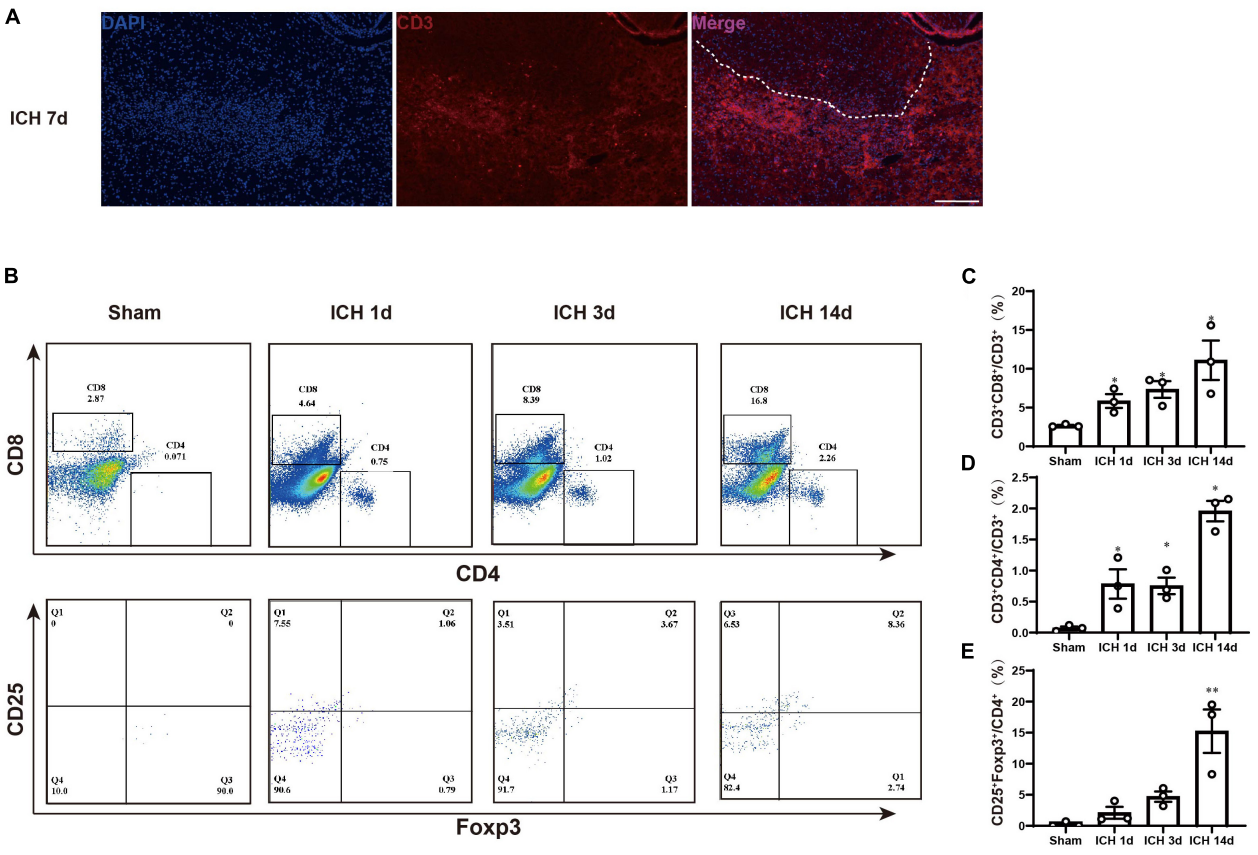

FIGURE 3 | T cell accumulation in the brain after ICH. **(A)** Immunofluorescence staining for CD3 in the hemorrhagic brain on 7 days after ICH. White dotted line indicates the hemorrhagic region. Scare bar = 200 μm. **(B–E)** Flow cytometric analysis of T cells in the hemorrhagic brain on 1, 3, and 14 days after ICH (n = 3 per group). Data are expressed as the mean ± SEM. $^*P < 0.05$. $^{**}P < 0.01$. $P < 0.001$ *vs*. sham group.

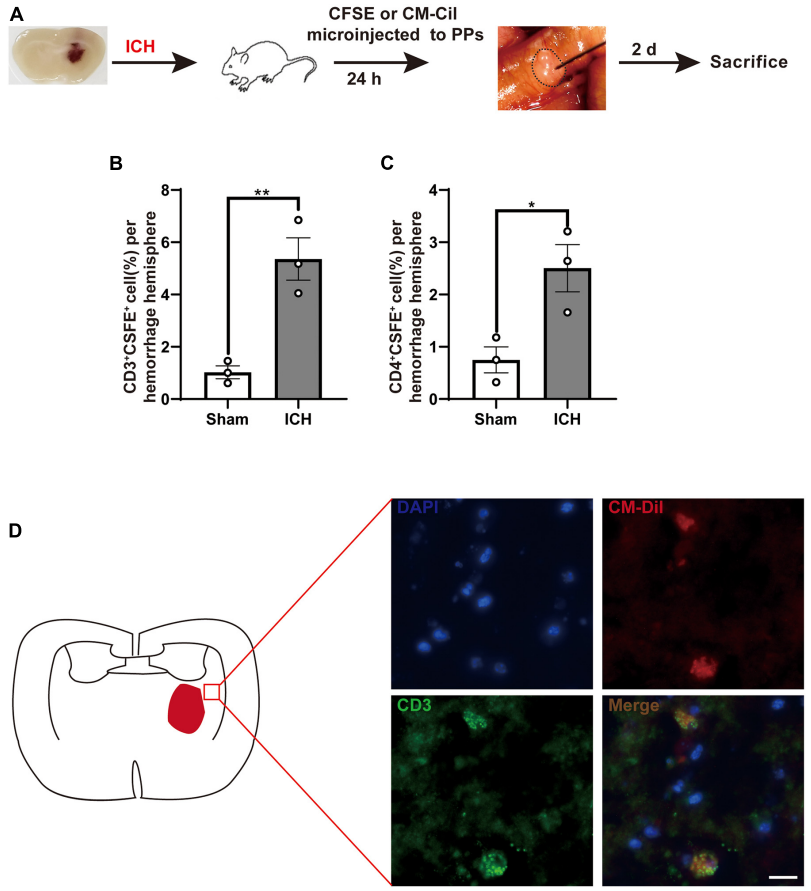

FIGURE 4 | Lymphocytes migrate from the intestine to the brain after ICH. **(A)** Experiment design for tracking the migration of intestinal PP-derived lymphocytes in mice after ICH. CSFE or CM-CII was microinjected into PPs 24 h after ICH. Two days later, the brain was dissected and analyzed for dye$^+$ T cells. **(B,C)** Flow cytometry analysis indicated an increased number of CSFE$^+$CD3$^+$ and CSFE$^+$CD4$^+$ in the hemorrhage hemisphere 3 days after ICH compared with sham group (n = 3). **(D)** Brain-invading CM-DiI T cells derived from intestinal PPs were identified in the peri-hematoma region and are illustrated as a cumulative map from three mice on one topographical coronal brain section at the bregma level. Data are expressed as the mean ± SEM. *P < 0.05. **P < 0.01 *vs*. sham group. Scale bar = 50 μm.

demonstrate that the invasion of many T cells from the intestine to the peri-hematoma region contributed to the ICH-induced neuroinflammation.

Fecal Microbiota Transplantation Reduces Neuroinflammation After Intracerebral Hemorrhage

Given the crucial role of T cells in ICH-induced neuroinflammation, we investigated whether FMT could affect the activity of T cells in the brain after ICH. qRT-PCR was used to examine the expression of the cytokines interleukin 17 (IL-17) and interferon-gamma (IFN-γ) and of the transcription factor Foxp3 as markers of different T_{helper} cells in brains 7 and 14 days after ICH. ICH + FMT group mice exhibited massively decreased expression of pro-inflammatory IFN-γ and IL-17 cytokines after ICH for14 days, which are markers of Th1 and Th17 T cells, respectively (p < 0.05, **Figures 5A,B**). However, Foxp3 expression, a marker of Treg cells, did not differ significantly between vehicle and ICH + FMT (**Figure 5C**).

In addition, we examined the temporal changes in the expression of pro-inflammatory genes in the brain after ICH. The messenger RNA expression levels of pro-inflammatory markers, including IL-1β, inducible nitric oxide synthase, and tumor necrosis factor-alpha, were significantly increased 14 days after ICH. However, this was reversed upon treatment with FMT (p < 0.05, **Figures 5D–F**). Nevertheless, their markers were not significantly different between the two groups after ICH 7 days (**Supplementary Figure 1**).

Frequent Fecal Microbiota Transplantation Ameliorated Intracerebral Hemorrhage-Induced Brain Injury

To assess the impact of healthy gut microbiota, we have first evaluated the weight changes in vehicle group mice and ICH + FMT mice (**Figure 6A**). After ICH, mice in our study

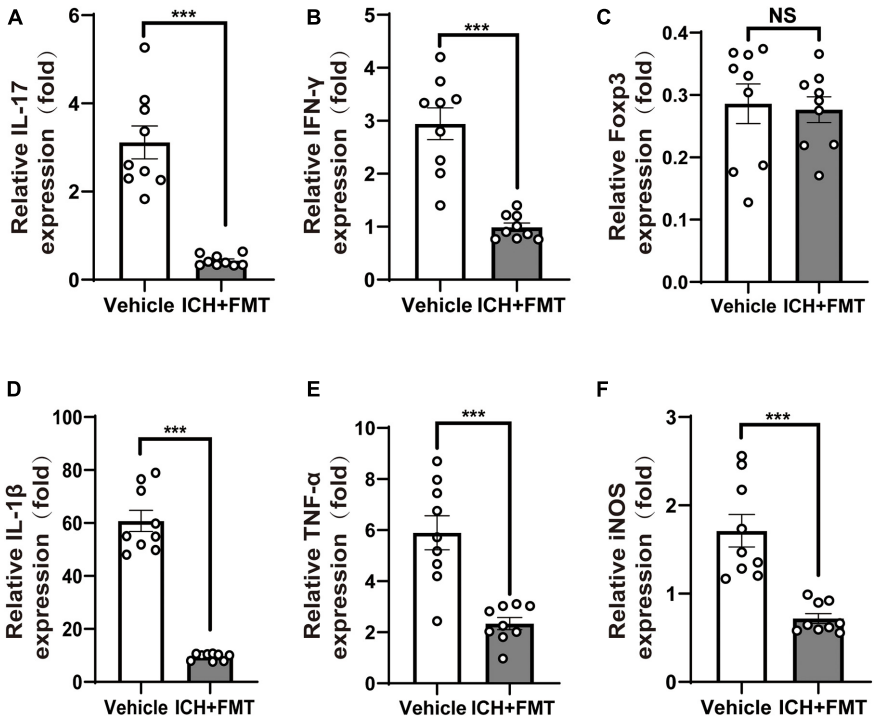

FIGURE 5 | Healthy fecal microbiome transplantation decreases neuroinflammation after ICH. **(A–C)** Recolonizing ICH mice with the gut microbiota obtained from healthy donor mice significantly decreased the gene expression level of IL-17 and IFN-γ compared with recipient of PBS ICH mice 14 days after ICH, but there was no significant change in the gene expression of Fxop3 (n = 3 per groups, 3 individual experiments). **(D–F)** Relative gene expression levels of IL-1β, tumor necrosis factor-alpha, and inducible nitric oxide synthase in the hemorrhagic basal ganglia. Recolonization with microbiota from healthy donor mice markedly suppressed IL-1β, tumor necrosis factor-alpha, and inducible nitric oxide synthase expression compared with ICH recipient of PBS ICH mice 14 days after ICH (n = 3 per groups, 3 individual experiments). Data are expressed as the mean ± SEM. ***$P < 0.001$ *vs*. vehicle group.

lost weight (≈13%) by day 3. By day 14, the ICH + FMT group recovered to their pre-ICH body weight (≈1%), whereas the vehicle group did not return to their original body weight (**Figure 6B**).

Next, we tested the method of FMT as a therapeutic approach to restore the healthy microbiome in animals after ICH. We performed a battery of behavioral tests, including the forelimb placing test, cylinder test, and corner turn test. All groups exhibited the same baseline of neurological function 3 days after ICH. In the forelimb placing test, the ICH + FMT group showed a significant improvement in the percentage of appropriate forelimbs compared with the ICH group on days 7 (5 ± 3.8% *vs*. 40 ± 7.1% in a vehicle), 14 (8.8 ± 3% vs. 51.3 ± 5.2% in a vehicle), and 28 (11.3 ± 3% *vs*. 66.3 ± 6.8% in a vehicle) (p 0.05, **Figure 6D**). In the cylinder test, the FMT group displayed a significant decrease in positive scores (compared to the vehicle group) on days 7 (75 ± 10.2% *vs*. 24.3 ± 9.5% in a vehicle), 14 (62.6 ± 8% *vs*. 26.1 ± 5.9% in a vehicle), and 28 (59.4 ± 4.1% *vs*. 15.8 ± 10.1% in a vehicle) ($p < 0.05$, **Figure 6E**). Unfortunately, we did not observe a significant difference in the corner turn test result (**Figure 6C**). Overall, our data indicated that restoration of healthy microbiota ameliorated the ICH-induced neurobehavioral impairment in the subacute phase and later phases.

Relationship Between Gut Motility Changes and Dysbiosis of Gut Microbiota

To explore the relationship between change in gut motility and gut microbiota dysbiosis. We used a surgical ileus mouse model to mimic the decreased gut motility patterns of ICH mice (**Figure 7A,B**). The gut microbiota composition was examined by 16S rRNA sequencing after surgery, and the sham mice revealed substantial changes 3 days after surgery (**Figure 7C**). We have also observed significant changes in the gut microbiota composition (**Figure 7D**). Our results suggest that the reduced gastrointestinal motility may be one reason for microbiota dysbiosis after ICH, although many factors can influence its composition.

Frequent Fecal Microbiota Transplantation Restores Intestinal Integrity After Intracerebral Hemorrhage

This was performed to assess the regulatory effect of FMT on the intestinal barrier after ICH. Compared with the sham group, the mean fluorescence intensity of the tight junction proteins Occludin and Claudin-1 was reduced after ICH but was restored after FMT treatment (**Figures 8A–C**). FMT treatment also reversed intestinal permeability barrier defects, as determined by the concentration of FITC-dextran in the plasma (**Figure 8D**).

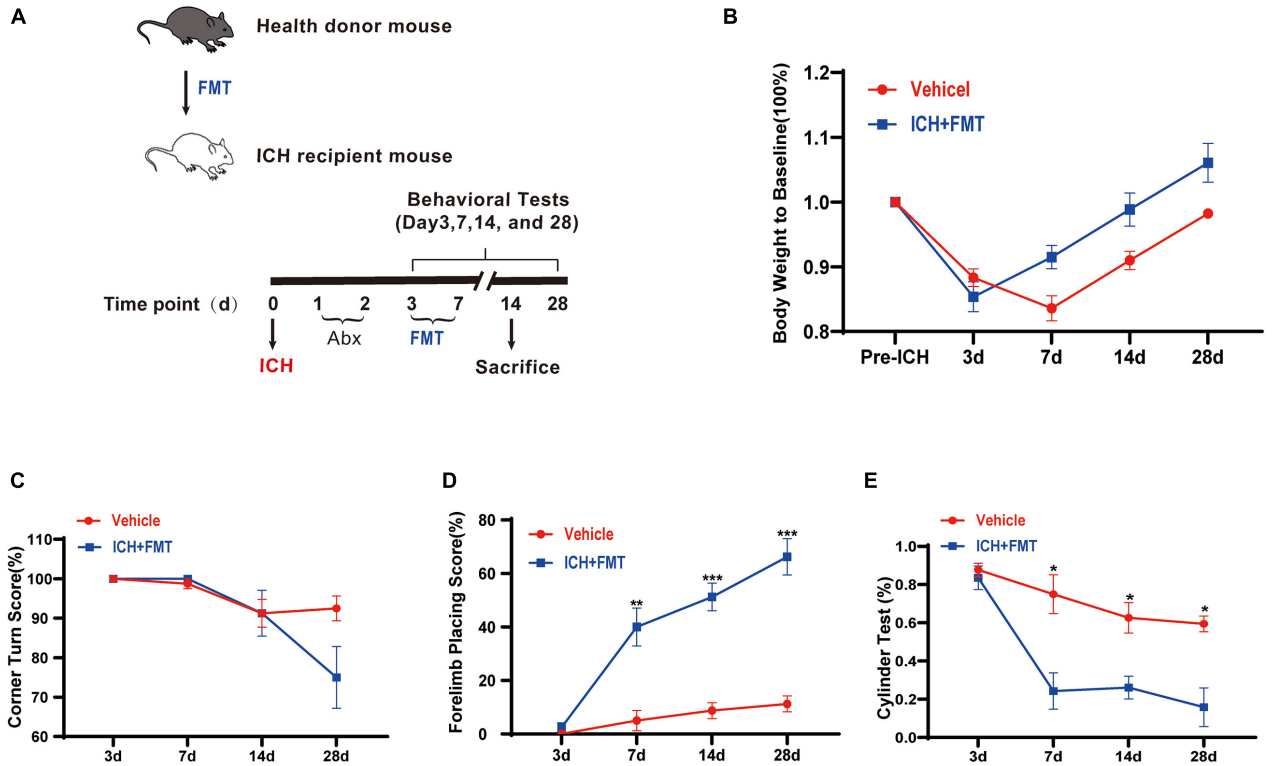

FIGURE 6 | Transplantation of healthy fecal microbiome improves neurobehavioral outcomes after ICH. **(A)** Experimental protocol for fecal microbiota transplant from healthy donor mice to recipient ICH mice. **(B)** Changes in body weight (n = 8). **(C–E)** Recolonizing ICH mice with the gut microbiota obtained from healthy donors significantly reduced behavioral performance as assessed by the corner turn test **(C)**, forelimb placing test **(D)**, and cylinder test **(E)** after FMT on 3, 7, 14, and 28 days after ICH. (n = 8 per groups, 2 individual experiments). Data are expressed as the mean ± SEM. $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$ *vs*. vehicle group.

DISCUSSION

In this study, we found that gut microbiota play an important role in ICH-induced neuroinflammation in mice. First, we demonstrated that ICH could induce gut microbiota dysbiosis, and this result is in agreement with previous studies in other acute CNS injuries (Benakis et al., 2016). Second, we also found obvious ICH-induced changes in gastrointestinal structure and function. Third, we found that the T cells have undergone dynamic changes after ICH, and in the acute phase of ICH, we found that the intestinal lymphocytes infiltrate into the perihematomal region. Fourth, transplantation of normal microbiota to ICH mice improved neurological outcome, and this effect is related to the attenuation of ICH-induced neuroinflammation. In addition, FMT treatment reduced intestinal barrier damage after ICH. Therefore, we can conclude that the gut microbiota is a key contributor that regulates neuroinflammation after ICH and that regulation of the composition of gut microbiota may be a possible therapeutic target for ICH.

Recent studies have shown that the gut microbiota is involved in the regulation of immune and inflammatory responses in acute and chronic neurological diseases. For instance, spinal cord injury increases intestinal permeability and bacterial translation from the gut, as well as exacerbates neurological impairment (Kigerl et al., 2016). In an ischemic stroke model, a large stroke lesion causes gut dysbiosis of the gut microbiota. In turn, gut microbiota dysbiosis impacts the immunity homeostasis and causes a pro-inflammatory response, leading to the deterioration of neurological stroke outcomes (Benakis et al., 2016; Singh et al., 2016). Patients with Parkinson's disease have obvious gut microbiota dysbiosis, which leads to an increase in the production of pro-inflammatory cytokines and a decrease in anti-inflammatory bacteria. Thus, gut microbiota dysbiosis is potentially related to Parkinson's disease state and progression (Liu et al., 2020). Alzheimer's disease causes gut microbiota imbalance, facilitating the infiltration of peripheral immune cells into the brain parenchyma and enhanced microglial activation, contributing to cognitive decline and amyloid-β burden in a mouse model of Alzheimer's disease (Kim et al., 2020). However, little is currently known about the changes seen in gut microbiota in ICH-induced brain injury.

Therefore, we explored changes in gut microbiota using a mouse ICH model. Our results show that ICH markedly alters the composition of the gut microbiota, as consistently seen in other CNS diseases (Singh et al., 2016). The diversity of microbiota species was markedly reduced, and gut bacterial changes were observed. As shown in a previous study, patients with ICH have obvious intestinal dysfunction (Cheng et al., 2018). Our results also suggested that ICH can reduce gastrointestinal motility (intestinal paralysis) and increase intestinal permeability

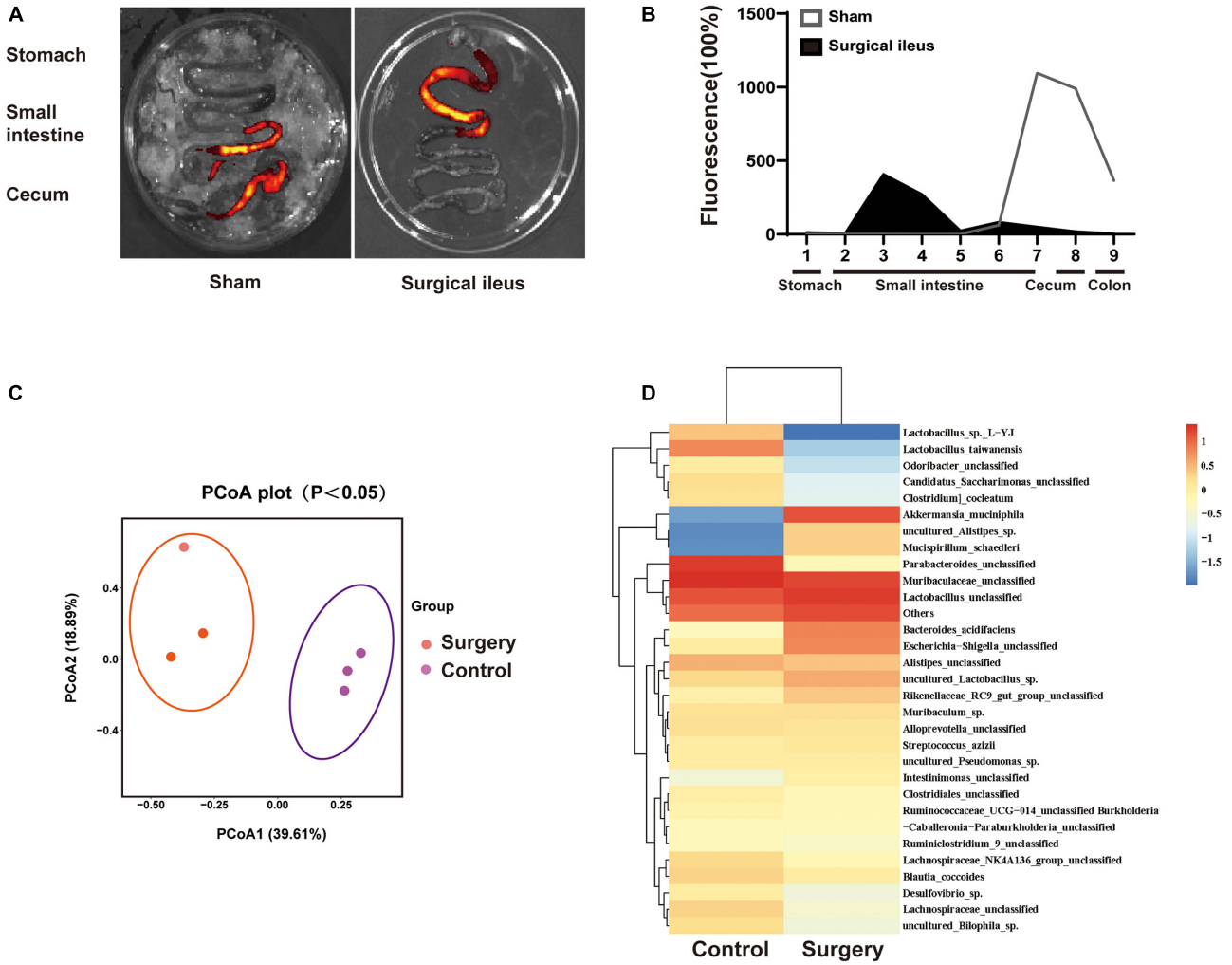

FIGURE 7 | Intestinal motility dysfunction and change in gut microbiota composition after intestinal paralysis. **(A)** Intestinal motility was measure 3 days after control and surgery ileus mice. **(B)** Representative fluorescence images of the complete gastrointestinal tract 1 h after gastric instillation of FITC-dextran showing retention of the fluorescence intensity in intensity segments after intestinal paralysis (n = 3 per group). **(C)** Principal coordinates analysis of the gut microbiota composition in control and intestinal paralysis in ICH mice after 3 days (n = 3 per group). **(D)** Heat maps of the cluster analysis showing gut microbiota composition in control and intestinal paralysis after 3 days mice (n = 3 per group).

in mice. ICH-induced brain injury also alters intestinal structure. In terms of the relationship between gastrointestinal paralysis and gut microbiota dysbiosis, the surgical ileus mouse model also showed significant gut motility decline and changes in gut microbiota composition. Therefore, we hypothesize that ICH-induced intestinal motility may be one reason for gut microbiota dysbiosis. Unfortunately, in our study, we could not declare any definitive causality.

Previous studies have suggested that complex immune and neuroinflammatory cascade responses are key factors in brain injury after ICH. ICH induces the activation of inflammatory cells and the release of cytokines, both of which exacerbate neuroinflammation and influence outcomes (Fu et al., 2021). Neuroinflammation induced by ICH is involved in many cellular and molecular processes (Wang and Dore, 2007). Cellular components include microglia, astrocytes, macrophages, and T cells (Tschoe et al., 2020). In healthy individuals, there are very few T cells in the brain (Hendrix and Nitsch, 2007). Previous studies have shown that cytotoxic T cells infiltrate the perihematomal region as early as 24 h and peaking after 2–7 days (Xue and Del Bigio, 2003). Zhou et al. (2017) found that $CD4^+$ T cells were increased 1 day and up to 14 days after ICH. In our study, we also found that T cells can infiltrate into the perihematomal region. The result showed that the $CD4^+$ T cells, $CD8^+$ T cells, and regulatory T lymphocytes (Tregs) were increased in the hemorrhagic hemisphere 1, 3, and 14 days after ICH. Considering that T cells are involved in ICH-induced neuroinflammation and undergo dynamic change, we hypothesized that T cells are important regulators of neuroinflammation after ICH.

Previous studies have suggested that gut microbiota dysbiosis can exacerbate the neuroinflammation in ischemic stroke

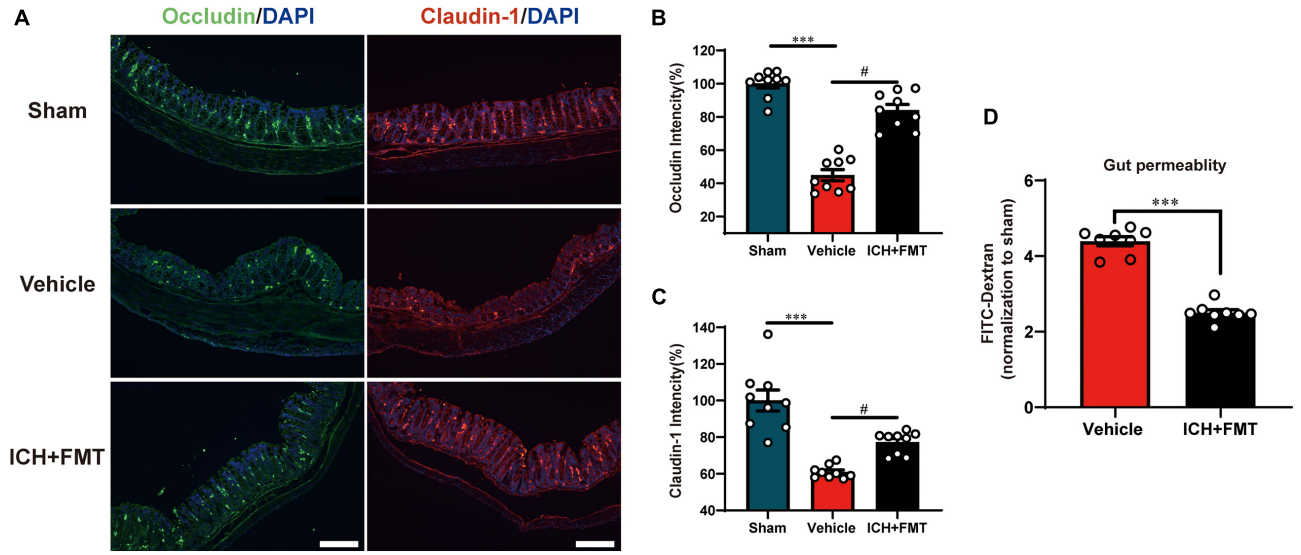

FIGURE 8 | Transplantation of healthy fecal microbiome improves gut intestinal integrity after ICH. **(A)** Representative immunofluorescence staining of the tight junction proteins Occludin and Claudin-1 in sham, vehicle, and ICH + FMT mice colons. **(B,C)** Mean of densities of Occludin and Claudin-1 (n = 3 per group, 3 individual experiments). **(D)** Intestinal permeability was improved after FMT treatment for 7 days (n = 4 per groups, two individual experiments). Data are expressed as the mean ± SEM. $^{***}P < 0.001$ *vs*. sham group. $^{\#}P < 0.05$ *vs*. vehicle group. Scale bar = 200 μm.

through alteration in T cell homeostasis. Restoring gut microbiota homeostasis may have beneficial effects in disease treatment (Singh et al., 2016; Kim et al., 2020). However, whether the gut microbiota regulates neuroinflammation after ICH through T cells is still unclear. Considering the essential role of gut microbiota in the regulation of neuroinflammation, we hypothesized that the gut microbiota is an important regulator of neuroinflammation after ICH. Our results demonstrated that gut microbiota dysbiosis is a key factor in influencing ICH-induced neuroinflammation and, thereby, neurological outcomes in ICH mice. The improved neurological outcomes observe after transplantation of healthy fecal microbiota to ICH mice clearly demonstrated a causal link between gut microbiota dysbiosis and changes in neuroinflammation. After ICH, the cytotoxic T cells were infiltrated into the perihematomal region, as evidenced by the increased levels of pro-inflammatory T cell markers IL-17 and IFN-γ. In line with previous research, our data have indicated that restoring the microbiota homeostasis by FMT significantly decreases the cytotoxic T cell infiltration after ICH, as evidenced by the decreased expression levels of pro-inflammatory cell markers IL-17 and IFN-γ, and can also decrease the levels of pro-inflammation cytokines, IL-1β, tumor necrosis factor-alpha, and inducible nitric oxide synthase. These results suggest that gut microbiota dysbiosis can induce cytotoxic T cell infiltration after ICH. In addition, FMT in ICH mice significantly alleviated ICH-induced secondary brain injury, thus contributing to improved neurological outcomes at 7–28 days after ICH. Moreover, we applied a cell-tracking experiment to intestinal PPs. At 3 days after ICH, the intestinal immune cells can invade into the areas of the brain with hematoma, which is consistent with previous reports of ischemic stroke (Singh et al., 2016). The results also strengthened the observation that gut microbiota regulates neuroinflammation after ICH through T cells.

After ICH, we found significant intestinal barrier damage as inferred from the concentration of FITC-dextran in the plasma. The intestinal barrier consists of three components: surface mucus, the epithelial layer, and immune defenses (Zhang et al., 2021). Tight junction proteins such as Claudin-1 and Occludin in the epithelial layer are essential for gut integrity (Saitou et al., 2000). In our study, we observed that after ICH, the mean fluorescence intensity of the tight junction proteins Occludin and Claudin-1 in the colon was reduced but was restored after FMT treatment. In addition, FMT treatment can decrease the concentration of FITC-dextran in mouse plasma after ICH. This result confirmed that gut microbiota has an impact on the gut barrier and that FMT can reduce intestinal barrier damage. However, the underlying mechanism still needs to be elucidated in future studies.

Although our work has provided evidence that restoring gut microbiota homeostasis attenuates neuroinflammation and improves the outcomes in ICH models in male mice, our study had several limitations. First, we did not elucidate the mechanism underlying the changes in gut microbiota in relation to ICH-induced cerebral immune responses. Further studies are required to elucidate the mechanism of action linking gut microbiota and neuroinflammation. Second, we have explored the effect of gut microbiota in male mice. However, sex and estrogen levels may affect ICH outcomes (Nakamura et al., 2005; Chang et al., 2020). Therefore, further studies are necessary to determine the effect of the gut microbiota in female mice after ICH. Third, age is an important factor that affects the gut microbiota and the functional outcomes of many CNS diseases (Spychala et al., 2018; Lee et al., 2020). However, in our study, we focused

on the role of gut microbiota in young mice rather than in older mice. Therefore, more studies are necessary to explore the role and underlying mechanism of gut microbiota in an older mouse model of ICH.

CONCLUSION

Our results indicate that significant gut microbiota dysbiosis after ICH contributes to neuroinflammation by affecting T cell homeostasis. In addition, FMT of healthy microbiota in ICH mice attenuates neuroinflammatory injury and improves neurological outcomes. Therefore, restoring gut microbiota homeostasis may minimize ICH-induced brain damage.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Ethics Committee of the Second Aliated Hospital, Zhejiang University of Medicine.

AUTHOR CONTRIBUTIONS

XF, JL, and GC conceived and designed the study. XY, GZ, HZ, and CX performed the ICH model and PCR. HZ, CX, BS, and FY performed the flow cytometry and immunostaining. LW and XF prepared the figures. XY and GZ analyzed data. XF, XY, and GZ prepared the manuscript draft. GC, LW, and FY wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.647304/full#supplementary-material

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frontiers in Immunology

REVIEW
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Intestinal Microbiota—A Promising Target for Antiviral Therapy?

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The intestinal microbiota is thought to be an important biological barrier against enteric pathogens. Its depletion, however, also has curative effects against some viral infections, suggesting that different components of the intestinal microbiota can play both promoting and inhibitory roles depending on the type of viral infection. The two primary mechanisms by which the microbiota facilitates or inhibits viral invasion involve participation in the innate and adaptive immune responses and direct or indirect interaction with the virus, during which the abundance and composition of the intestinal microbiota might be changed by the virus. Oral administration of probiotics, faecal microbiota transplantation (FMT), and antibiotics are major therapeutic strategies for regulating intestinal microbiota balance. However, these three methods have shown limited curative effects in clinical trials. Therefore, the intestinal microbiota might represent a new and promising supplementary antiviral therapeutic target, and more efficient and safer methods for regulating the microbiota require deeper investigation. This review summarizes the latest research on the relationship among the intestinal microbiota, anti-viral immunity and viruses and the most commonly used methods for regulating the intestinal microbiota with the goal of providing new insight into the antiviral effects of the gut microbiota.

Keywords: COVID-19, SARS-CoV-2, intestinal microbiota, virus, immunity

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INTRODUCTION

The intestinal microbiota has important regulatory effects on both innate and adaptive immunity. A variety of viruses have been verified in animal studies and clinical trials to disrupt the intestinal microbiota, leading to the aggravation of immune disorders, more severe symptoms and further infection (1–3). The role of the intestinal microbiota has interested scholars since the discovery of its potential links to overall health. Faced with the worldwide epidemic caused by SARS-CoV-2, *The Diagnosis and Treatment Protocol of COVID-19* (the 7th tentative version) issued by the China National Health Commission mentions that intestinal microecological regulators can prevent secondary bacterial infections by maintaining intestinal microecological balance, and it emphasizes the importance of the intestinal microbiota balance in antiviral immunity, making the intestinal microbiota a focus of anti-epidemic strategies.

Conditional pathogenic or exotic viruses in the gut inevitably encounter the intestinal microbiota during the process of invading the body, and the intestinal microbiota has been demonstrated by many experiments to promote or inhibit the invasion of specific viruses in the intestine (4). The intestinal microbiota can even affect antiviral immunity in extraintestinal organs and tissues to a certain extent (5) through the so-called "gut-lung axis" (6), "gut-liver axis" (7) and "gut-brain axis" (8). Recent studies have reported high expression of the SARS-CoV-2 receptor angiotensin-converting enzyme 2 (ACE2) on differentiated enterocytes (9) and successful viral replication in the intestinal epithelium (10), suggesting that the intestine might be another viral target organ. Consistent with these findings, clinical trials have also shown that patients with gastrointestinal symptoms have a worse prognosis (11), indicating that the intestinal microbiota may affect clinical outcomes in patients infected with SARS-CoV-2 to a certain extent by regulating the immune status of the lung or intestine and even through direct interaction with viruses.

This review summarizes the latest findings regarding the possible relationship among the intestinal microbiota, anti-viral immunity and viral infection and some of the most commonly used methods of intestinal microbiota regulation to provide a new theoretical basis and molecular strategy for controlling viral infections as well as more effective and safer methods for bacterial regulation and for identifying effective targets.

THE REGULATORY EFFECTS OF INTESTINAL MICROBIOTA ON IMMUNITY

Studies have shown that the intestinal microbiota plays an important role in modulating the immune system against viruses (12–15). The regulatory effects of the intestinal microbiota on viral infection are closely intertwined with local and systemic immune responses and contribute to both congenital and adaptive immune responses (16, 17). The intestinal microbiota may prevent or promote viral infections, primarily *via* bacterial components, metabolites and regulating the immune response of the host (18–20).

Short-chain fatty acids (SCFAs) are the most indispensable metabolites of the intestinal flora, including acetic acid, propionic acid, and butyric acid. SCFAs reduce the growth and adhesion of pathogenic microorganisms, improve the integrity of the epithelium, and further enhance systemic host immunity by reducing the intestinal pH, thus increasing the production of mucin (21, 22). SCFAs activate G protein-coupled receptors (GPCRs) and inhibit histone deacetylase (HDAC) to exert their biological functions (23). According to a study by Trompette A et al., SCFAs also regulate the haematopoietic function of Ly6c(-) patrolling monocytes, enhance the function of CD8 T cells, and activate GPR41 to provide protection against influenza virus infection (20). In addition to SCFAs, there are many other metabolites of the intestinal flora that are reportedly related to host immunity. Pyruvate and lactate, which are produced by the intestinal flora, help to enhance immune responses by inducing the growth of GPR31-mediated CX3CR1+ dendrites in the gut (24). Research by Steed A et al. showed that desaminotyrosine (DAT), a metabolite of the intestinal flora, protects against influenza by increasing type I IFN signalling in macrophages (25).

Toll-like receptors (TLRs) are pattern recognition receptors (PRRs). In innate immunity, TLRs recognize pathogen-associated molecular patterns (PAMPs). TLRs can recognize bacterial flagellin and single-stranded viral RNA to mediate antiviral and antibacterial immune responses (26, 27). Influenza virus infection significantly increases the mRNA expression of TLR7 in lung immune cells. Antibiotic-induced dysregulation reduces the expression of genes involved in the TLR7 signalling pathway, while probiotic intervention restores the initial expression upregulation of genes, such as TLR7 (28). Furthermore, the microbiota composition critically regulates the generation of virus-specific CD4 and CD8 T cells and antibody responses after influenza virus infection (29). The intestinal flora plays an essential role in the maintenance of immune homeostasis by strengthening the integrity of the barrier functions of the gut mucosa, which is an important aspect of systemic immunity (30, 31). Moreover, the healthy intestinal flora plays a crucial role in regulating TLR 7 signal transduction, which has been found to mitigate common mucosal immune system (MIS) damage caused by antibiotic treatment in mice (28).

In addition, many researchers are studying how the gut microbiome affects immunity in distal parts of the body, such as the lungs, brain and liver. For instance, changes in the microbial community in the lungs, including the airways, can affect the composition of the intestinal flora. In addition, some gastrointestinal diseases are also associated with alterations in the respiratory tract (32). The transduction of immunomodulatory signals and the transfer of metabolites between the lungs and gut constitute the gut-lung axis (33). The intestinal and respiratory mucous membranes provide physical barriers to microbial penetration, and the colonization of the normal microbiome is resistant to pathogens. Bacterial transfer from the gastrointestinal tract to the lungs has been observed in sepsis and acute respiratory distress syndrome, in which barrier integrity is impaired (34, 35). The gut-brain axis refers to the two-way information network between the intestinal flora and the brain. In the gut, segmented filamentous bacteria can restore the functions of B and T lymphocytes (36). T lymphocyte receptors (TLRs) are also widely distributed on neurons (37). Therefore, gut epithelial cells transport viral and bacterial metabolites to the inner environment, neurons respond to microbial components, and the nervous system interacts with these bacterial and viral components. The balance of the intestinal flora may alter the regulation of the inflammatory response and may take part in regulating emotion and behaviour (38, 39). Because the liver is exposed to gut-derived microbial metabolites and components, intestinal dysbiosis is involved in liver disease, inflammation, and fibrosis (40). The gut-liver axis is also associated with autoimmune liver diseases, such as primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) (41).

In conclusion, the intestinal microbiota is capable of influencing organismal immunity locally and systemically, proximally and distally. Studying the possible mechanism by which the intestinal flora regulates host immunity can provide a clearer understanding of the occurrence and development of diseases.

VIRUSES CAN CHANGE THE COMPOSITION OF INTESTINAL MICROBIOTA

Despite a lack of clinical trials, many viruses that can spread by faecal-oral transmission and primarily induce gastrointestinal symptoms have been revealed to impact the composition of the intestinal microbiota. Novel duck reovirus (NDRV), a subtype of reovirus, was shown to result in the loss of SCFA-producing bacteria and the compensatory expansion of pathogenic bacteria in poultry (42, 43). Porcine epidemic diarrhoea virus (PEDV) is another diarrhoea-related pathogen with the ability to disrupt the intestinal microbiota balance, resulting in an increased abundance of *Escherichia* and *Clostridium* (44). Changes in the intestinal microbiota induced by rotavirus are correlated with changes in physiological parameters, such as white blood cell counts and blood urea nitrogen, in neonatal calves (45). No experiments have explored the mechanisms of these changes induced by viruses. We speculate that intestinal microbiota imbalance might be a by-product of intestinal epithelial injury since these infections always cause both morphological and functional damage.

Interestingly, some viruses that are not considered to be directly involved in intestinal epithelial injury can affect the components of the intestinal microbiota.

A study of 15 COVID-19 patients in Hong Kong showed that infection with SARS-CoV-2 significantly altered the faecal microbiomes of all 15 patients, which manifested as an enrichment of opportunistic pathogens and a depletion of beneficial bacteria in patients compared to healthy individuals, and the imbalance of intestinal microbiota persisted after SARS-CoV-2 clearance (46). This finding reveals that the abundance of certain species, such as *Coprobacillus* and *Clostridium ramosum*, is correlated with COVID-19 severity (46). Consistent with the findings in Hong Kong (47), a subsequent study in which shotgun sequencing was performed on the total DNA extracted from stools from COVID-19 patients also showed a low proportion of gut microbiota with immunomodulatory potential, including *Faecalibacterium prausnitzii*, *Eubacterium rectale* and bifidobacteria. The exact mechanism by which SAR-CoV-2 infects the intestinal microbiota is not clear. COVID-19 could cause patients to experience a state of severe inflammatory stress with increased secreted proinflammatory cytokines, such as TNF-α and IL-6 (48, 49), in blood and tissues. As important mediators of inflammation in the gastrointestinal tract (50), cytokines might result in intestinal inflammation and disrupt the homeostasis of the intestinal microbiota. High expression of ACE2, the receptor of SARS-CoV-2, was recently observed in the intestinal epithelium. Although currently no evidence supports the ability of SARS-CoV-2 to invade the host through the ACE2 in the gastrointestinal tract, the possibility that SARS-CoV-2 alters the composition of the intestinal microbiota through this type of route cannot be excluded.

Other respiratory viral infections also exhibit potential for remodelling intestinal microbiota.

As early as 2014, Wang et al. (51) reported that during influenza infection, lung-derived CCR9+ CD4 T cells can be recruited to intestinal tissues and enhance the proportion of *Escherichia coli* (*E. coli*) by generating IFN-γ. Excess *E. coli* results in IL-15 overexpression, which stimulates the differentiation of CD4 T cells into Th17 cells that damage the intestine (51). Infection by respiratory syncytial virus (RSV) was also demonstrated to disrupt intestinal microbiota homeostasis (1). Recently, Groves et al. (52) found that similar changes in gut microbiota composition occur in response to RSV and influenza A virus infection and are accompanied by common symptoms, such as weight loss and inappetence. An increased ratio of *Bacteroidetes* to *Firmicutes* abundance, which is associated with reduced calorie intake (53, 54), was observed in this research as well, suggesting that inappetence might be an important cause of the changes in the gut microbiota after respiratory viral infections.

Intestinal microbiota imbalance is also common in HIV infection and is likely attributed to persistent inflammation, the direct effects of antiretroviral drugs and even HIV virions (55).

DUAL REGULATORY EFFECT OF THE INTESTINAL MICROBIOTA ON VIRAL INFECTION

Possible Mechanisms That Facilitate Viral Infection

As shown in **Figure 1**, intestinal microbiota might facilitate viral invasion through different mechanisms.

Binding to Viruses

Bacterial lipopolysaccharide (LPS), a product on the exterior surface of gram-negative bacteria, binds to and primes the signalling of its relatively specific receptor (TLR4) to initiate an appropriate or excessive immune response (56). Recently, polysaccharides, of which LPS is the most representative member, were found to bind with several enteric viruses and were positively correlated with the enhanced environmental stability of several viruses. Research conducted by Kuss et al. (57) demonstrated that poliovirus incubated with gram-negative or gram-positive bacteria exhibited significantly increased viability and infectivity, which was mediated by binding to N-acetylglucosamine (GlcNAc)-containing polysaccharides, especially LPS, and specific bacteria, such as *Bacillus*. The same results were observed in another study (58) in which LPS exposure stabilized the capsid against chlorine bleach at high temperatures and delayed its RNA release, suggesting that

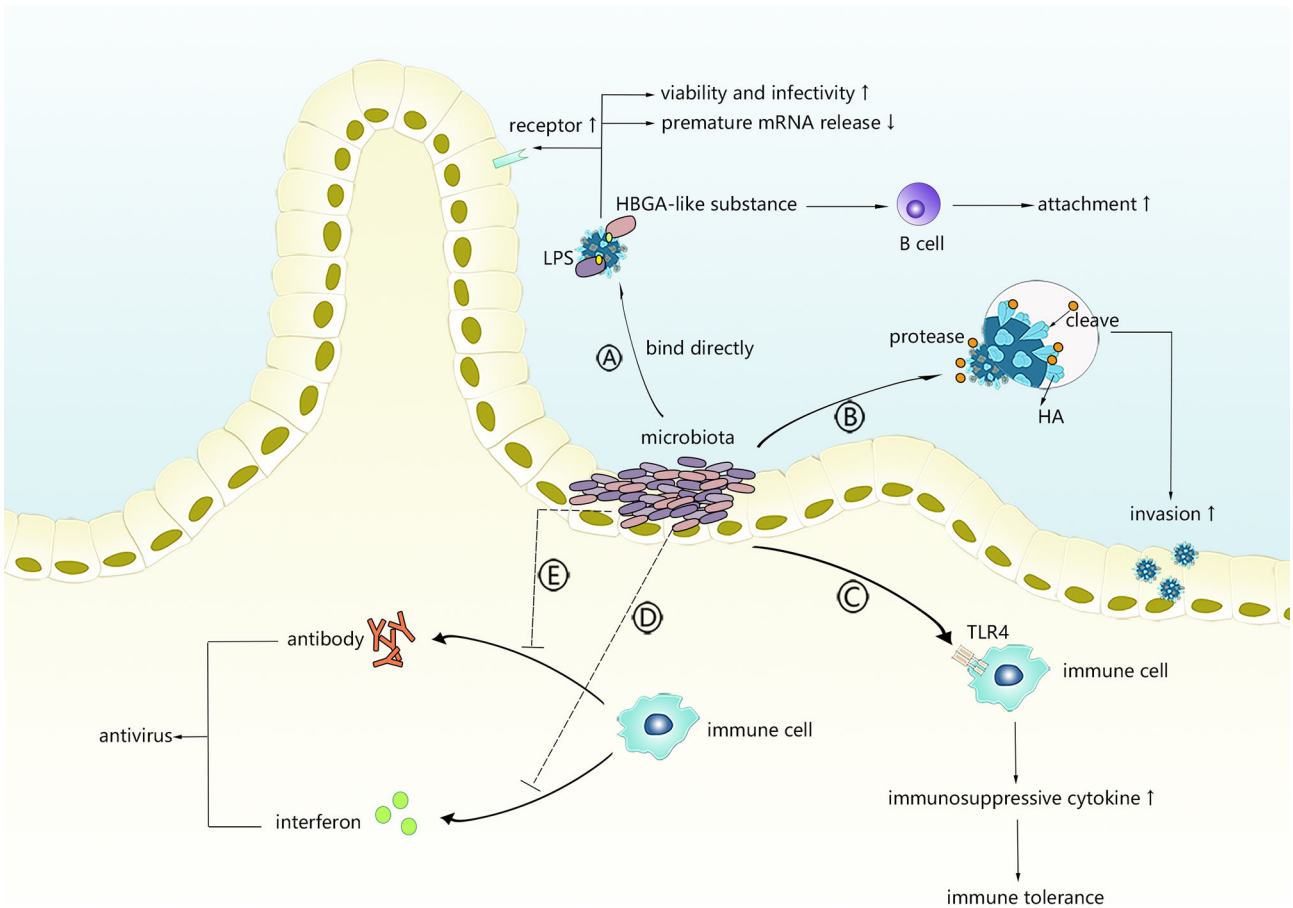

FIGURE 1 | The possible mechanism of intestinal microbiota promoting virus infection. Intestinal microbiota can directly interact with viruses or regulate innate immunity or adaptive immunity. **(A)** Viruses bind with LPS or HBGA-like substances derived from intestinal microbiota. **(B)** Intestinal microbiota secretes protases to activate viruses. **(C)** Intestinal microbiota primes TLR-4 signalling to induce immunosuppressive microenvironment. **(D, E)** Intestinal microbiota interferes with production of antiviral antibodies or interferons.

binding to LPS might stabilize the particles by limiting premature RNA release. Another result demonstrated that VP1-T99K, a mutated strain of poliovirus with a reduced LPS binding ability, showed relative instability when added to faeces (58) and verified the facilitative effects of LPS on virion stability. LPS was also shown to strengthen the binding of poliovirus to its receptor, which could partly explain why LPS enhances the attachment of poliovirus to host cells. Mammary tumour virus (MMTV) and reovirus are two additional viruses that could benefit from interacting with LPS to increase stability and attachment. Using negative stain transmission electron microscopy, Berger et al. (59) observed that the direct interaction of bacterial outer envelope components with virions mediates reovirus thermostability and infectivity, while the specific binding residues remain unclear. Findings showed that for both virions and intermediate reovirus particles (ISVP), lipoteichoic acid and N-acetylglucosamine-containing polysaccharides enhanced their thermostability, which could translate into enhanced attachment and higher infectivity instead of reovirus use of its proteinaceous cellular receptor junctional adhesion molecule-A or cell entry kinetics in a serotype-dependent manner, providing evidence that the interaction of viruses with the intestinal microbiota can aid infectious agents through enhanced biophysical properties of the virion that translates into enhanced infectivity. MMTV was demonstrated to express LPS-binding factors, such as CD14, TLR4 and MD-2, which are conducive to having LPS binding protein (LBP) on the envelope (60). Direct viral binding to LPS is thought to be the mechanism underlying facilitation in MMTV infection, in which LPS could help stabilize the virus and then prime TLR4 signalling, inducing the production of immunosuppressive cytokines that prolong the persistence of MMTV (60).

Host histo-blood group antigens (HBGAs), including ABO/H, secretor and Lewis families, are recognized as receptors by numerous viruses, including noroviruses (NoVs), rotaviruses (RVs), and coronaviruses. Some intestinal flora produce HBGA-like substances, and enteric bacteria might directly bind some viruses and affect viral invasion. Miura et al. (61) first revealed that HBGA-like substances localize on the extracellular

polymeric substances (EPS) of human enteric bacteria to capture HuNoVLPs and play a key role in binding to NoVLPs. The MuNoV titre and attachment of GII.4 to intestinal B cells are reduced by the antibiotic-induced depletion of normal intestinal flora before oral infection (62). However, incubation with H-type HBGA-expressing *E. cloacae* or H antigen resulted in dose-dependent infectivity restoration, whereas neither *E. coli* without H antigen expression or LPS could rescue infectivity, confirming that HuNoV interactions with HBGA-like substances could facilitate the infection of and attachment to B cells. In another study involving microbiota-depleted mice infected with murine norovirus (MNV), faecal virus shedding was significantly decreased, while the transplantation of faeces from untreated mice restored MNV CR6 infectivity (63), suggesting that the intestinal microbiota plays an important role in persistent norovirus infection.

The results also provide insight into norovirus infection therapy, showing that molecules that possess a binding capacity to HBGA-like substances might alleviate noroviral infection by competing with virions for intestinal receptors.

However, the effects of the intestinal microbiota on viral attachment can be completely reversed by different experimental strategies. To investigate how specific bacteria influence NoV attachment to host cells, Rubio and colleagues performed competitive exclusion experiments (binding assays in the presence of bacteria), exclusion experiments (incubation with bacteria followed by incubation with P-particles) and displacement experiments (incubation with P-particles followed by incubation with bacteria) on P-particles (P-particles were structured by purified P-domains from NoV genotypes GI.1 and GII.4 and maintained their ability to bind to host receptors) and several bacteria expressing HBGA-like substances on their surface (64). In both the exclusion and displacement experiments, probiotic and non-probiotic bacteria exerted positive effects on P-particle attachment, whereas both kinds of bacteria were shown to have a negative effect on P-particle attachment in the competitive exclusion experiments. We speculate that HBGA-positive bacteria might be able to block virion attachment in suspension by reducing the available binding sites on virions, whereas other interactions, such as the binding of virions to bacteria, might lead to higher virion retention on the surface of the host cells. The regulatory effect of the intestinal microbiota on the immune microenvironment and intestinal mucosa glycosylation can also alter the stability, retention and infectivity of viruses that bind to the intestinal microbiota.

Regarding the impact on virion stability, HBGA-norovirus interactions were shown to protect noroviruses against abiotic stresses (65), but these protective effects were not observed for attachment to HBGA-positive *E. coli* or Tulane virus (66). Further studies are needed to elucidate the role of specific interactions between human norovirus and environmental matrices in virus thermal stability.

Secretion of Proteases

Cleavage of haemagglutinin (HA) mediated by proteases is essential for cell entry by receptor-mediated endocytosis during the process of influenza virus invasion. During the previous century, several bacteria were verified to have the ability to activate the influenza virus by cleaving HA by directly secreting or increasing the synthesis of proteases (67, 68). King et al. (69) examined isolates of the cloacal microbiota and found several protease-secreting bacteria and a variety of proteases, indicating that specific intestinal microbiota, such as *Enterococcus faecalis* and *Proteus mirabilis*, might alter the pathogenicity of influenza viruses with the help of proteases and facilitate viral invasion.

Induction of an Immune-Tolerant Microenvironment

PAMPs from commensal flora rather than pathogens are generally recognized by TLRs in the intestine, and the intestinal epithelium seems to tolerate the presence of commensal bacterial PAMPs, which usually do not provoke an inflammatory immune response (70). According to previous studies, the existence of intestinal commensal bacteria induces both enteric T-regs (71) and peripheral generation of Tregs (72) to limit indiscriminate inflammatory responses. Thus, viruses might take advantage of intestinal commensal bacteria by binding to their surface or products to escape the antiviral immune response. TLR4, a specific signalling receptor of gram-negative bacterial LPS, induces immune tolerance under continuous stimulation with low-dose LPS and has been shown to exert a negative effect on viral clearance and antiviral immunity in some cases.

MMTV, an enveloped retrovirus that expresses LPS-binding proteins, requires commensal and functional TLR4 bacteria to maintain persistence (73, 74). Through LPS receptors integrated in the viral envelope, such as CD14 and MD-2 (60), the virus cloaks itself in bacterial LPS and activates TLR4, leading to the production of immunosuppressive cytokines and the blockage of the antiviral response (74). MNV infection was shown to be mediated by intestinal bacteria through a similar mechanism (75). Norovirus, which does not generally cause obvious intestinal inflammation, provokes inflammatory lesions in IL10-deficient mice. The generation of inflammation requires enteric microbiota since intestinal lesions are not observed in germ-free IL10-deficient mice. Based on these findings, inducing the production of inflammatory suppressive cytokines, such as IL-10, by intestinal flora might represent a possible evasion mechanism against the antiviral immunity of MNV.

Interference With Interferon Production

Interferons are cytokines with critical importance to innate immune regulation in antiviral immunity, among which IFN-λ has been shown to have potent antiviral effects against multiple viruses, such as rotavirus, reovirus and norovirus. Both exogenous and endogenous IFN-λ were shown to inhibit the replication of specific viruses effectively in the intestine in animal experiments. Viral dependence on commensal bacteria and sensitivity to IFN-λ were first linked in a study on MNoV conducted by Baldridge and colleagues (63). Commensal microbiota depletion was shown to prevent persistent MNoV infection in wild-type mice, while infection was established in microbiota-deficient mice lacking Ifnlr1, Stat1 and Irf3, which

are important factors for IFN-λ induction or signalling pathways, suggesting that commensal bacteria might promote the persistence of MNoV infection by decreasing antiviral responses mediated by IFN-λ (63). Through subsequent experiments, IFN-λ was revealed to have an obvious ability to establish MNoV infection independent of the adaptive immune response, which is generally thought to be required for viral clearance (76). Another interesting finding of this study is that MNoV replication was detected in haematopoietic cells, whereas IFN-λ acted on non-haematopoietic cells, suggesting that IFN-λ does not directly act on infected cells but rather exerts indirect regulatory effects. In addition to facilitating viral replication, certain enteric bacteria have the potential to promote organ damage secondary to viral infection through the IFN-λ pathways. *Helicobacter hepaticus*, which is more likely to colonize the colon under HBV infection, was found to act with some specific innate lymphoid cells (ILCs) to indirectly activate the IFN-γ/p-STAT1 axis, generating a detrimental immune microenvironment and accelerating the tumorigenesis of HCC (hepatocellular carcinoma) (77). Other enteric viruses, such as echovirus 11, enterovirus 71 and avian influenza virus, induce IFN-λ, and these findings suggest that viral infection can be indirectly controlled *via* the regulation of intestinal flora IFN-λ.

Interference With Antibody Production

Intestinal bacteria might reduce the immunosuppressive effects of viruses by interfering with the production of antiviral antibodies. By assessing rotavirus infection and replication and measuring the humoral responses of wild-type mice and microbiota-depleted mice in the days after rotavirus infection, Uchiyama et al. (78) demonstrated that rotavirus antibody levels in the microbiota-depleted group were significantly higher than those in the controls within the first few weeks. Although the antibody levels between the two groups were similar in the ninth week, the total IgA and IgG levels in microbiota-depleted mice were markedly higher, indicating that commensal microbiota promote RV infection by partially blocking the production of RV-specific antibodies.

Based on the fact that antibodies in acute HIV-1-infected individuals are predominantly targeted to HIV Env gp41 and cross-reactive with commensal bacteria, Trama et al. (79) hypothesized that these bacteria are ineffective in inducing antibodies against viruses, promoting the persistence of HIV infection. There is a normal subset of B cells that are reactive to intestinal commensal bacteria in memory B cell pools in the intestine. When facing HIV infection, the body might send out memory B cells that recognize the activation of the intestinal flora and control bacteria, such as *E. coli*, instead of native B cells, which induce specific HIV antibodies because the gp41 region of the HIV capsid is similar to the antigen of *E. coli*. As a result, non-neutralizing antibodies directed at Env gp41 are generated, and the restrictive effects of humoral immunity on HIV are alleviated. Intestinal commensal bacteria are very large, forming a complicated biological complex that is the source of many PAMPs, which induce a large number of memory cells that are generally tolerated by the immune system. Once viral components possess a similar conformation to bacterial antigens, specific bacteria might facilitate the virus through a similar mechanism as in HIV infection.

Possible mechanisms by which the microbiota facilitates viral infection are briefly summarized in **Table 1**.

Possible Mechanisms of Viral Infection Inhibition

As shown in **Figure 2**, microbiota can alleviate viral infections within and outside the intestinal tract through numerous regulatory mechanisms.

Stimulation of Cell Turnover

Commensal bacteria, especially gram-positive bacteria, are able to stimulate the proliferation, migration and turnover of intestinal epithelial cells (IECs) by generating SCFAs (82, 83). Recently, epithelial cell turnover induced by bacteria was revealed to potentially confer protection against certain enteric viral infections and diarrhoea. Shi et al. (84) unexpectedly discovered that the presence of unique segmented filamentous bacteria (SFB) not only protected mice from RV infections and related diarrhoea but also reduced their susceptibility to reovirus, vesicular stomatitis virus, and influenza A viral infections *in vitro*. Assessments of the transcriptional response in the

TABLE 1 | Possible mechanism by which the intestinal microbiota promotes viral infection.

Methods	Mechanisms	Viruses	Reference
Binding to viruses	Increasing viral stability by LPS	Poliovirus	(57, 58)
	Facilitating viral invasion by HBGA-like substances	Reovirus	(59)
		MMTV	(60)
		HuNoV	(61)
		MuNoV	(63)
Secreting proteases	Activating viruses	Influenza virus	(67, 69)
Inducing immune tolerance microenvironment	Priming TLR-4 signalling	MMTV	(73, 74)
	Inducing production of inflammatory suppressive cytokines	MuNoV	(75)
Interfering with interferon production	Downregulating antiviral response mediated by IFN-λ	MuNoV	(63, 76)
	Indirectly activating IFN-γ/p-STAT1 axis	HBV	(77)
Interfering with the production of antibodies	Blocking the production of a specific antibody	Rotavirus	(78)
	Inducing an invalid antibody	HIV	(79)
Generating metabolites	Inducing poor CD4 T-cell reconstruction through butyrate	HIV	(80)
	Suppressing the expression of ISG	Influenza virus	(81)

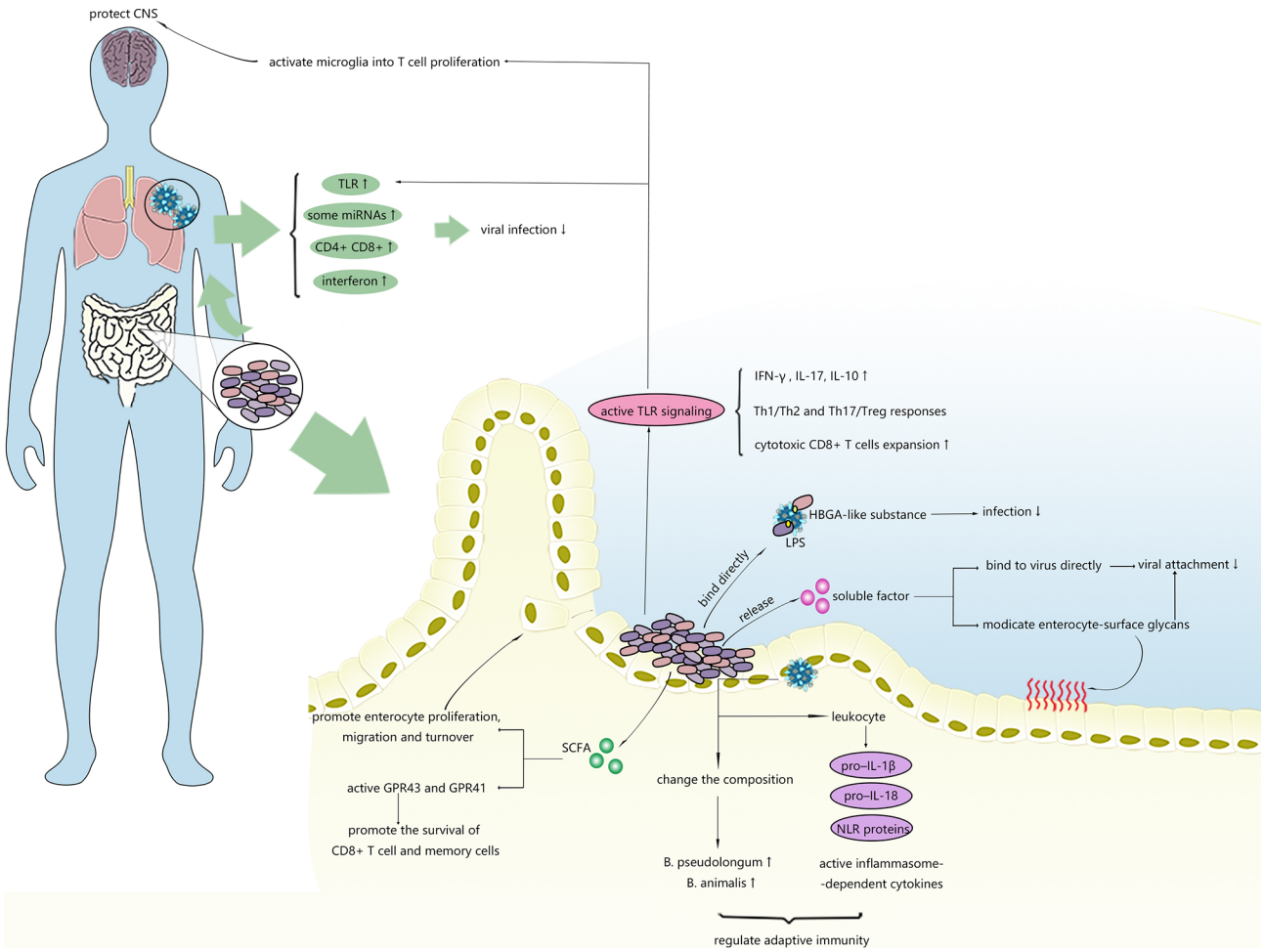

FIGURE 2 | Possible mechanisms of intestinal microbiota inhibiting virus infection. Intestinal microbiota can regulate the immune response in the gut and distal tissues and affect stability of viruses.

intestinal epithelium showed that SFB-treated mice expressed transcripts related to pathways involved in cell turnover rather than traditional intestinal immune-mediated mechanisms, unlike untreated mice. The effects of SFB in accelerating epithelial cell turnover were also supported by observations of the increased proliferation and migration of ileum cells and the slight elongation of villi, suggesting that the stimulatory effect of commensal bacteria on host cell turnover and renewal may result in unexpected antiviral effects and might provide new targets for the treatment of RV infection or other enteric viral infections.

Binding to Viruses

Although most virions that bind to the intestinal microbiota exhibit increased thermal stability and stronger attachment to host cells, in some cases, the binding of viruses to the surface of bacteria might inhibit viral infection rather than promote it.

As discussed previously, LPS was previously found to bind to viruses, such as poliovirus and MMTV, resulting in increased cell attachment, persistent infectivity at elevated temperatures and increased immune evasion and transmission of the viruses (58, 60). However, influenza A virus (IAV), which is transmitted primarily *via* the faecal-oral route in wild birds, exhibits reduced stability when incubated with LPS, long-term persistence and the freeze-thaw stability of distinct HA subtypes from different host origins (85). Bandoro et al. (58) hypothesized that LPS might interact with and constrict the lipid envelope of IAV or bind to other domains of HA, except for the receptor binding site, to trigger conformational changes in HA to confer protective effects against viral fusion to host cells.

A variety of enteric bacteria have been found to express HBGA-like substances, the receptor for numerous viruses, such as norovirus and RVs. Previous studies have shown that bacteria-virus binding *via* HBGA-like substances facilitates viral invasion (62). Subsequently, relevant studies on hNoV surrogates (P particles) *in vitro* have shown that the binding of HBGAs and specific viruses serves as both an inhibitor or a promoter of viral infection in different situations (64). In pigs with HBGA-expressing *E. cloacae* colonization, which is expected to facilitate HuNoV invasion, infectivity was inhibited, and the data suggested that *E. cloacae* blocked the attachment of viral

particles (86). HBGA-expressing probiotic bacteria were shown to alleviate viral infection through a similar mechanism (87). The inhibitory effects on cellular attachments of viruses *via* bacteria-virus interactions may also occur for EcN and HRV (88), since EcN was observed to primarily interact with VP4, which serves as a major viral cell attachment protein of HRV in gnotobiotic piglets. Moreover, the cellular attachment of HRV and HRV shedding were significantly reduced. The variable effects of the intestinal microbiome on viral attachment and invasion might be caused by differences in binding sites for viruses, interference with viral attachment to epithelial cells or even immunostimulatory effects on both the systemic and intestinal immune systems. The specific mechanisms by which the microbiota facilitates RV and NoV viral infection remain unclear. Furthermore, further research on whether binding to HBGA-like-coated bacteria or free HBGA-like substances aids viruses in productive attachment or stabilizes the viruses before reaching the infection sites *in vitro* and *in vivo* is needed. Experiments on P-particles and hNoV surrogates also demonstrated that specific microbiota might have the ability to inhibit hNoV invasion by binding virions in some cases.

The underlying mechanisms by which the microbiota promotes RV and NoV viral infection are far from being understood. It is also unknown whether binding to HBGA-coated bacteria or free HBGA participates in the entry process during infection, helping viruses to perform productive attachment, or just allowing the viruses to reach their infection sites.

Regulation of Immune-Related MicroRNAs

The beneficial effects of the intestinal microbiome on antiviral immunity are not limited to the gastrointestinal tract. Prophylactic consumption of probiotic bacteria successfully shortened the duration and reduced the severity of respiratory viral infections in clinical trials (89). It has been demonstrated that the expression of antiviral defence genes and responsive pathways in macrophages can be altered by intestinal dysbacteriosis, leading to failed control of viral infection and increased host morbidity and mortality (90). MicroRNAs (miRNAs) are considered key for interdomain molecular communication between the host and gut microorganisms. However, the role of microRNA communication in antiviral immunity regulation remains unclear. In a study conducted by Pang et al. (91), intestinal dysbacteriosis caused a decrease in miR-29c that played an antiviral role in lung tissues and led to enhanced pulmonary influenza virus amplification. Once the specific communication mechanism between the intestinal microbiota and lungs is fully understood, regulating the gut flora might be likely to help improve diseases.

Priming of TLR Signalling

Transmembrane cell receptors are essential for identifying pathogens in innate immunity, among which up to 10 subtypes of Toll-like receptors (TLRs) have been identified in humans. TLR7, which can recognize ssRNA from the influenza virus (92), was shown to be negatively regulated in antibiotic-treated mice after respiratory influenza viral infection, along with reduced downstream cytokines, such as IFN-γ and IL-17, and they disrupted the balance between Th1/Th2 and Th17/Treg responses (28). Immune impairment and TLR downregulation can be rescued by TLR7 ligand or the restoration of the intestinal flora, indicating that the intestinal microbiota provides protection against influenza infection by increasing the activity of the Toll-like receptor 7 (TLR7) signalling pathway. TLR7 also plays a critical role in the recognition of other viruses, such a HIV and vesicular stomatitis virus (92). Therefore, we speculate that the intestinal microbiota might also assist with viral control in infection with these viruses *via* the same mechanism.

Mounting studies have verified that clearing HBV requires mature intestinal microbiota (93–95). Evidence has revealed that exposure to low levels of microbe-derived LPS activates TLR4-mediated IL-10 secretion, eliciting liver tolerance that facilitates the persistence of HBV infection (93). Nevertheless, bacterial CpG-DNA (a TLR9 ligand) overrides liver tolerance through CpG-DNA/TLR9, increasing the expansion of HBV-specific cytotoxic CD8 T cells and leading to virus clearance (96). The prohibitive or permissive effects of the intestinal microbiota on HBV infection might depend on the strength and type of signals derived from bacteria. In addition to stimulating TLRs, the intestinal microbiota can also activate GPR43 and GPR41 by releasing SCFAs to promote the survival of CD8 T cells and memory cells (94).

The regulatory effect of the intestinal microbiota on the immune system *via* TLR signalling has recently been verified to be effective in preventing damage to the central nervous system (CNS) following viral infection. Brown et al. (97) recently demonstrated that products derived from the intestinal microbiota were sufficient to activate microglia for T cell proliferation in the CNS, providing aid against JHMV infection through microglia-intrinsic TLR4 signalling.

Regulation of Adaptive Immunity

The risk of respiratory syncytial virus infection is increased in infants with reduced exposure to the intestinal microbiota. Ichinohe et al. (29) revealed a correlation between influenza virulence and intestinal microbiota diversity. They noted that antibiotic-treated mice failed to mount both innate and adaptive immune responses against influenza virus infection, and this immune dysregulation was associated with the deficient generation of CD4 and CD8 T cells in lung tissue, while the local or distal injection of Toll-like receptor (TLR) ligands restored lung immunity against the influenza virus. Furthermore, loss of immunoregulation did not occur in antibiotic-treated mice infected with herpes simplex virus type 2 (HSV-2), immunity against which does not require inflammasome activation (29). These results suggest that the products of specific symbiotic bacteria trigger multiple pattern recognition receptors, stimulating leukocytes, which release factors that can support the production of pro–IL-1β, pro–IL-18, and NLR proteins, providing signalling for the activation of inflammasome-dependent cytokines. Consistent with what Ichinohe et al. (97) observed, broad-spectrum antibiotic treatment also reduced the generation of influenza-specific antibodies and T cells, weakening the ability of mice to clear the influenza virus.

Anaerobic bacteria are thought to be the primary strains modulating immune responses against influenza in the lungs by suppressing the adaptive immune response in the lungs and reducing proinflammatory cytokines, such as IFN-γ and IL-17. The anti-influenza function of anaerobes was confirmed by a recent study (98) in which a transplantation of faecal microbiota from surviving mice previously infected with virulent influenza increased the resistance of the recipient mice to influenza, indicating that the faecal material contained specific intestinal microbes with protective effects against influenza. The results further showed that the presence of *B. pseudolongum*, *Lactobacillus*, and *B. animalis* was closely correlated with survivability, and the abundances of these specific bacteria were associated with responses to influenza infection in addition to the responses of the initial gut microbes (98). Based on these findings, Zhang et al. (29) hypothesized a mechanism in which the gut microbiota might increase the abundance of endogenous *B. pseudolongum* and/or *B. animalis* to enhance the resistance of the host to influenza infection. The results of a functional metagenome analysis indicated that *B. animalis* may provide protection against influenza by promoting the biosynthesis of specific amino acids, such as valine and isoleucine, exhibiting protective effects against influenza, proposing a hypothesis regarding the mechanism underlying the protective effects of the intestinal microbiota against the influenza virus and first reporting the anti-influenza effects of *B. animalis*. Further research is needed to verify the authenticity of and specific molecular regulatory pathways involved in this hypothesis. Together, these findings suggest a protective role of intestinal bacteria in mediating the host immune response to influenza.

The above results were all derived from mouse models, and Yitbarek et al. (99) extended these findings to other species by confirming the critical role of the intestinal microbiota in controlling the H9N2 subtype of Avian Influenza Virus (AIV). As first-line innate immune factors, type I IFN levels increase after H9N2 infection in chickens, leading to the upregulation of IFN-stimulated genes and subsequent antiviral responses. The antibiotic-induced depletion of the intestinal microbiota impairs type I IFN responses in lung tissue, the gastrointestinal tract and the trachea (99). These results combined with the finding that double-stranded RNA of specific commensal intestinal microbiota has the ability to induce basal levels of type I IFNs suggest that the intestinal microbiota might initiate anti-H9N2 influenza responses *via* type-I IFN-dependent mechanisms. The expression of IL-22, which can assist in viral infection control, along with IFNs *via* IFN receptor signalling and STAT1-dependent pathways, was significantly downregulated in antibiotic-depleted chickens and was subsequently restored by treatment with probiotics or faecal microbiota transplantation (FMT), suggesting that IL-22-related mechanisms also take part in anti-H9N2 influenza immunity mediated by the intestinal flora.

The intestinal microbiota might also take part in antiviral immunity in other viral infections in addition to influenza virus infection. The antibiotic-induced depletion of the intestinal microbiota prior to LCMV infection induces physiological changes that include impaired adaptive immunity in mice, such as decreased titres of LCMV-specific IgG and the expansion of LCMV-specific CD8 T cells (90). In mice treated with oral antibiotics, susceptibility to flavivirus infections, such as severe West Nile (WNV), Dengue, and Zika virus, increases, T cell responses are impaired, and the levels of WNV-specific CD8 T cells are decreased (14). Taken together with the findings related to influenza, these results suggest that the intestinal microbiota might affect extra-gastrointestinal tract viral infections by diminishing the adaptive immune response.

Regulation of Glycosylation Changes on the Intestinal Surface

Numerous previous descriptive clinical studies have shown that the application of probiotics is effective at shortening the duration of viral diarrhoea or reducing rotavirus shedding (100). Soon after, their secreted soluble factors are considered effective and considerably safer for the host (101). In a study conducted by Jolly et al., RCA lectin strongly inhibited infection by both human and animal rotavirus strains in host cells (102). Subsequent studies also verified the involvement of this sugar in viral adhesion by binding with the spike protein of rotaviruses (103). In 2012, Varyukina et al. first demonstrated that bacteria-derived soluble factors that increase cell-surface galactose led to the blockage of rotavirus infections (104), indicating that modifications of intestinal epithelial cell-surface glycans caused by bacteria-derived soluble factors prevent RV attachment.

Antiviral Effects of Intestinal Microbiota Products

The human intestinal microbiota converts the nutrients in food into a variety of metabolites, the accumulation of which in the bloodstream regulates both local and distant immune responses. These metabolites exert metabolic and signalling functions similar to those of the metabolites of pharmaceutical agents. SCFAs, bacterial metabolites derived from the metabolism of soluble fibres by specific microbiota, were shown to improve gut homeostasis by activating GPCRs such as GPR41, GPR43, or GPR109a (105) and inhibiting histone deacetylases (106). Recently, components of SCFAs were revealed to be closely related to antiviral immunity against certain viruses. The intake of dietary fibre, which is a raw material for SCFA production in pregnant women, was demonstrated to provide protection against severe RSV in new-borns (107). Protection against rotavirus infection conferred by a high-fibre diet was also observed in animal experiments. Antunes et al. (108) demonstrated that acetate is a key protective metabolite in RV infection that helps reduce viral load and pulmonary inflammation *via* a distinct mechanism by which acetate promotes the responses of type 1 interferon and the expression of interferon-stimulated genes in lung epithelial cells by activating GPR43.

Desaminotyrosine (DAT) is another intestinal bacterial metabolite that was recently demonstrated to enhance the expression of multiple type I IFN-stimulated genes (ISGs) in the lung tissues of influenza-infected mice but conferred no

benefit to animals lacking in the expression of immunity-related guanosine triphosphatase family M member 1 (Irgm1) when used alone as a treatment (25). These results combined with the findings that infection with the influenza virus results in poorer outcomes in antibiotic-treated or germ-free mice (29) suggest that certain components of the intestinal microbiota prime type I IFN affect signalling and exert distal effects on responses to influenza viruses producing DAT (25).

Dozens of metabolites may contribute to adverse complications in virus-infected patients. Some components of the intestinal microbiota, such as *Anaerococcus*, *Clostridium*, *Escherichia*, *Proteus*, *Providencia* and the *Edwardsiella* genus, help break down dietary phosphatidylcholine and are partially responsible for the production of choline, carnitine, betaine, and trimethylamine N-oxide, which are independently correlated with cardiovascular complications (109, 110). According to multiple previous studies, chronic HIV infection significantly alters the intestinal mucosa and microbiota (111), resulting in the enrichment of bacteria belonging to the genus *Prevotella* (110) that are thought to play a certain role in generating the four metabolites mentioned above. Recently, Sinha et al. (112) demonstrated that intestinal disturbances caused by HIV infection significantly enhance the levels of carnitine-related metabolites and are closely related to adverse cardiovascular events in patients. For diseases related to viral infections, studies on the correlation between gut-derived phosphatidylcholine metabolites and adverse cardiovascular complications have primarily been concentrated on HIV. Nevertheless, many other viral infections, including HCV infection (113), enhance the risk of developing coronary artery disease. Gut dysbiosis in HCV infection manifests as an acceleration of the proinflammatory microbiota (114), which might induce the production of metabolites that accelerate the development of atherosclerosis. With further study, identifying the community structure of the intestinal microbiota may be a promising method for risk assessment regarding cardiovascular diseases during viral infections.

Another product of the intestinal microbiota, butyrate, which is an SCFA, exerts contradictory effects during viral infections. Experiments have shown that butyrate contributes to the health of distant organs, such as the lungs (115). Lee et al. verified a negative correlation between butyrate-producing gut (BPG) bacteria and a risk for lower respiratory viral infections in kidney transplant recipients (116). The same results were also reported in patients undergoing allogeneic haematopoietic stem cell transplantation (117). However, for many other viral infections, BPG bacteria seem to facilitate viral infection and aggravate the development of infection. Enrichment of *F. prausnitzii*, unclassified *Subdoligranulum* sp. and *C.comes*, which have the ability to produce butyrate in HIV-1-infected individuals, is associated with poor CD4 T-cell reconstruction (18), and sodium butyrate acts as an inducing agent of Epstein-Barr virus (EBV) reactivation (80), as shown *in vitro*. Butyrate was also verified to promote cellular infection with the influenza virus, reovirus, HIV-1, human metapneumovirus, and vesicular stomatitis virus (81). Detections of related genes have shown that butyrate significantly suppresses the expression of specific antiviral IFN-stimulated genes (ISGs) by reprogramming the type I IFN-mediated innate antiviral immune response, revealing a new mechanism by which butyrate influences viral infections of cells (81).

Unclear Effects

Adenoviruses (AdVs) are the primary pathogens that cause severe diarrhoea in children and represent major viral pathogens in immunocompromised adults (118), which could result in intestinal microbiota imbalance *via* the disruption of epithelial cells (119). A recent study revealed that a dysfunctional intestinal microbiota could make an individual more susceptible to disease-causing AdV infections (120). However, the mechanism remains unclear.

The possible mechanisms by which the microbiota suppresses viral infection are briefly summarized in **Table 2**.

COMMONLY USED METHODS TO REGULATE THE MICROBIOTA

Probiotics

Probiotics contain microbiota strains of lactic acid bacilli and specific non-pathogenic *E. coli* that provide beneficial properties to the host. Probiotics show great potential for treating or preventing viral-related diseases, especially respiratory virus infections and viral gastroenteritis. A new systematic review showed that ingesting probiotics improves the clinical symptoms of viral gastroenteritis, such as the duration of diarrhoea and hospitalization, suggesting that probiotics should be administered to patients with viral gastroenteritis (121). The use of probiotics is also recommended for HIV-infected patients. An increasing number of clinical trials have demonstrated that probiotics confer certain curative effects with respect to improving gastrointestinal symptoms, increasing CD4 counts and sometimes reducing the plasma HIV load in HIV(+) children, adults and even infants (122–124). Evidence-based medical research has shown that with the assistance of prebiotics, probiotics significantly increase CD4 counts, especially in females (125), which is likely a result of restoring intestinal CD4 T-cells induced by epithelial healing.

The role of probiotics in respiratory tract virus infections has been continuously examined, but high-quality evidence has not yet been produced to verify their curative effectiveness.

Previous evidence has demonstrated that probiotic administration might reduce the risk of viral upper respiratory illness, but the quality of the evidence is very low (126), and the efficacy of probiotics must be further verified. Probiotic ingestion successfully reduced the risk of influenza infection and other respiratory viral infections by 35% in people in long-term and chronic care facilities compared to the placebo group, but the results were not significant (127). In a randomized controlled trial of 152 seronegative volunteers who received a challenge from rhinovirus type 39, administering *Bifidobacterium animalis* subspecies lactis Bl-04 significantly reduced the CXCL8 response to rhinovirus infection but had no influence on subjective symptom scores, infection rate or respiratory inflammation (128). Oral ingestion of Bl-04 also appeared to interfere with

TABLE 2 | Possible mechanisms by which intestinal microbiota inhibit viral infection.

Methods	Mechanisms	Viruses	Reference
Stimulating Cell turnover	Suppressing viral invasion	Rotavirus	(84)
Binding to viruses	Decreasing virus stability *in vitro* through LPS	Influenza virus	(85)
	Blocking virus attachment *via* HBGA-like substances accompanied by other unclear mechanisms	HuNoV Rotavirus	(86, 87) (88)
Regulating the immune-related microRNAs	Increasing miR-29c production in lung tissue	Influenza virus	(91)
Priming TLR signalling	Upregulating the toll-like receptor 7 (TLR7) signalling pathway	Influenza virus	(28)
	Upregulating the toll-like receptor 9 (TLR9) signalling pathway		(96)
	Priming microglia-intrinsic TLR4 signalling	HBV JHMV	(97)
Regulating adaptive immunity	Increasing generation of CD4 and CD8 T cells	Influenza virus	(29)
	Increasing the abundance of endogenous *B. pseudolongum* and/or *B. animalis*		(98)
	Upregulating IFN-stimulated genes	Influenza virus	(99)
	Increasing specific CD8 T cells		(14)
		Influenza virus Flavivirus	
Regulating glycosylation changes on the intestinal surface	Modifying epithelial cell-surface glycans through bacteria-derived soluble factors	Rotavirus	(104)
Secreting bacterial metabolites	Increasing interferon-stimulated gene expression by generating acetate	Rotavirus	(108)
	Priming type I IFN signalling by DAT	Influenza virus	(25)
Unclear mechanisms		AdV	(119, 120)

rhinovirus replication, as manifested by reduced viral shedding in nasal secretions (128). These results indicate that probiotics have the potential to alter the baseline state of innate immunity and the subsequent host response to rhinovirus infection, whereas even though virus-specific CCR5+ effector memory CD4 T cells were found to be critical members in controlling rhinovirus (129), a probiotic modulation of T-cell populations or broader immune signatures in rhinovirus infection has not yet been observed. In a subsequent trial, neither rhinovirus infection nor oral probiotic consumption affected the abundance of the nasal microbiota but did influence clinical symptoms during rhinovirus infection, and the administration of probiotics through the nasal cavity might be used to treat rhinovirus-associated diseases or respiratory viral infections effectively (130).

Based on current clinical trials, we speculate that probiotic administration might be more effective in alleviating virus-related illnesses that are more likely to alter the composition of the intestinal microbiota. The species specificity of the effects of probiotics on immune function might also be an influencing factor. In conclusion, probiotics are currently recommended for treating viral gastroenteritis and HIV infection. The effects of probiotics against other viral illnesses require further verification in larger samples.

FMT

FMT is the procedure by which microorganisms from the fresh or frozen faecal matter of healthy donors are directly transferred to a patient, and this technique has been primarily adopted for treating recurrent *Clostridium difficile* infection (131) and has been widely studied since it was approved by the U.S. Food and Drug Administration in 2003 to treat *Clostridium difficile* infection. The effects of FMT have also been shown in other gastrointestinal (GI) diseases and non-GI diseases (132).

FMT is a promising microbiota-modulating therapy for HBV- or HCV-related diseases. Administering FMT (a faecal suspension containing *Lachnospiraceae* and *Ruminococcaceae*) was observed to restore microbial diversity and function in the intestine and reduce serious adverse events in HCV-derived cirrhosis patients administered a 5-day broad-spectrum antibiotic treatment (133). A decreased ratio of *Bifidobacteriaceae*/*Enterobacteriaceae* and the translocation of intestinal bacterial products contribute to the development of HBV infection in asymptomatic carriers, chronic patients and decompensated cirrhosis patients infected with HBV (134). Therefore, FMT seems to be a promising new therapy for HBV-related illness due to its ability to reverse the proportion of certain specific bacteria in the intestine.

A recent study reported that FMT induces hepatitis B virus e-antigen (HBeAg) clearance in patients with HBeAg. In this study of 18 HBeAg-positive patients who were taking antivirals for more than 3 years, 3 out of 5 patients treated with FMT presented HBeAg clearance, while none of the 13 patients who did not receive FMT treatment exhibited HBeAg clearance (135). FMT also reduced serum HBeAg titres after each session (135). Two additional studies also verified the curative effects of FMT in clearing HBeAg; in one study, the HBsAg titres decreased after each FMT session and the serum endotoxin levels decreased (136), and in the other study, FMT resulted in a 16.7% HBeAg clearance rate in patients with chronic hepatitis B (137). In addition to having potential curative effects, FMT is a relatively simple and short-duration treatment that likely costs less than traditional repeated antiviral therapy.

Since diarrhoea induced by intestinal microbiota imbalance is an important cause of death in HIV-infected individuals, the

possibility of using FMT to alleviate HIV-related illness is also of significant concern. A pilot study meant to assess the safety and efficacy of FMT in HIV infection showed that FMT application was associated with increased levels of peripheral Th17 and Th22 cells and benefitted intestinal T cell activation with no observed adverse effects (138), suggesting that FMT might represent a potential therapy for restoring T cell subset homeostasis in HIV-infected patients. However, although the latest systematic review reported that the efficacy and safety of FMT are nearly the same in patients with and without intact immunity, the safety concerns cannot be ignored since the heterogeneity of immunosuppressive subtypes makes the responses to FMT in single or combined immunocompromised states unpredictable (139).

Another issue is that the reshaping of the microbiome community structure by FMT does not last long (138), indicating that supplementary methods might be needed to maintain the remodelling of the intestinal microbiota and help with the colonization of exotic bacteria.

Antibiotics

Antibiotics are the cornerstone of anti-infective drugs and maintain human health by targeting pathogens. Some commensal microbiota, however, might be affected more or less by antibiotic administration, especially broad-spectrum antibiotics. The overuse, prolonged use or incorrect use of antibiotics can bring up some unanticipated and undesirable consequences, including the intestinal domination of pathogenic bacteria, transient or profound loss of both microbial species and microbial diversity, increased and prolonged susceptibility to infection and the risk of reoccurring infection (140). Broad-spectrum antibiotic administration led to a significant reduction in *Bacteroidetes* and a concurrent increase in *Firmicutes*, the two groups of microbiotas that dominated over 90% of the gut communities (141). Infancy is usually considered a critical period for intestinal flora establishment due to its low diversity and the poor stability of gut microbiota compared to adults. Lu et al. found that β-lactam, a kind of antibiotic typically used in new-borns with infectious diseases, significantly reduced the overall diversity of the gut microbiota and the abundance of some beneficial bacteria, such as *Bacteroides*, in the new-borns while increasing the abundance of some opportunistic pathogenic bacteria, such as *Enterococcus* (142). Vrbanac et al. investigated the effects of ampicillin and vancomycin on the gut microbiota and metabolome and found that the local abundance of ampicillin and its metabolites was not only correlated with a loss of alpha diversity but was also related to an increased metabolome effect size. Small peptides from host proteins, including histones, were increased in the lower gastrointestinal tract of mice after treatment with these two antibiotics (143).

Fortunately, the native microbiota has a degree of self-recovery ability, and after a period of time, its composition and function will be close to those of the pre-treatment state (144). For example, frequent antibiotic administration in the NICU initially delays the maturation of the preterm neonatal microbiome, but the gut microbiota achieves a similar composition as that of antibiotic-naive term controls by 15 months of age (145). α-Defensins, the most abundant antimicrobial proteins of the intestine, are crucial for the replenishment of *Bacteroides* from the mucosal reservoir by promoting their colonization following microbiota dysbiosis induced by antibiotics (146). Although the resilience of the intestinal flora ensures that it can recover as much as possible after being disturbed by antibiotics, specific species and antibiotic-resistance genes (ARGs) still distinguish those treated with antibiotics from healthy controls (147). The abundance of ARGs increases markedly during antibiotic treatment, and the abundance of those that are chromosomally encoded decreases after antibiotic withdrawal, while the abundance of other ARGs that are episomally encoded persists for much longer periods of time (148).

There are still many cases in which a history of antibiotic therapy was more associated with the development of some diseases. A case-control study of Kawasaki disease (KD), including 50 patients and 200 control subjects, showed that the development of KD was associated with previous antibiotic administration and that antibiotics might contribute to the development of KD by affecting the intestinal microbiota in infants and young children (149). Additionally, long-term antibiotic exposure has been associated with an increased risk for several diseases, such as type 2 diabetes (150), inflammatory bowel diseases (151), and asthma (152). Therefore, the interaction of antibiotics and intestinal microbiota must be taken into account when administrating antibiotics.

Traditional Chinese Medicine (TCM)

Given the potential risk of administering conventional medications, such as antibiotics, TCM has attracted increasing interest for many disease treatments, such as diabetes (153), ulcerative colitis (UC) (154) and kidney diseases (155), the mechanisms of which have been further demonstrated to be associated with the intestinal microbiota. The treatment of diabetes mellitus is one of the most typical examples showing that TCM's regulatory effect on the intestinal flora exerts a therapeutic role (153, 156). Berberine (BBR), which is extracted from a traditional Chinese herb, is used to alleviate symptoms of type 2 diabetes mellitus. Yao et al. reported that the richness and diversity of gut microbiota in type 2 diabetes rats treated with BBR showed increasing trends compared to untreated rats (153). The same mechanisms were also found during the treatment of antibiotic-associated diarrhoea (AAD) with Xianglian pill (XLP), a traditional Chinese pharmaceutical preparation synthesized from BBR as a raw material (157). *Centella asiatica* (CA) is a traditional medicinal herb with a long history of anti-inflammatory application that was demonstrated to reshape the gut microbiota in UC mice by increasing the α-diversity and shifting the community by depleting colitis-associated genera to repair the intestinal mucosal barrier (154). The Baitouweng (BTW) decoction also improved inflammatory symptoms in mice with UC by modulating the intestinal microflora, including decreasing

the proportion of *Firmicutes* to *Bacteroidetes* and the ratio of *Proteobacteria*, decreasing the relative abundance of *Escherichia-Shigella* and increasing the relative abundance of *Lactobacillus* and *Akkermansia* (158).

Many signalling pathways might be involved in the TCM-mediated treatment of UC through the intestinal microbiota. Kuijieyuan decoction (KD), a traditional Chinese medicine, alleviates intestinal barrier injury in ulcerative colitis, exerting antioxidant and anti-inflammatory properties by affecting TLR4-dependent PI3K/AKT/NF-κB signalling (159).

Qing et al. found that the IL-6/STAT3 pathway was suppressed by BTW treatment, resulting in a better curative effect (158). Qingchang Suppository (QCS) and its ingredients are capable of downregulating the levels of IL-6 and STAT3 in LPS-induced Caco-2 cells and of alleviating the symptoms of trinitrobenzenesulfonic acid (TNBS)-induced colitis in rats, suggesting that the JAK2/STAT3 pathway might also be a potentially involved signalling pathway. Although the exact mechanisms by which TCM improves diseases through the intestinal microbiota require further exploration, there is no doubt that TCM exerts potential therapeutic effects.

DISCUSSION

An increasing number of studies have indicated both direct and indirect (through the immune system) mutual regulation between the intestinal microbiota and viruses. Restoring intestinal microbiota homeostasis by using probiotics, FMT or the antibiotic-induced depletion of intestinal microbiota can affect the duration and severity of specific viral infections, as mentioned earlier. In cirrhotic patients with viral hepatitis, the restoration of microbial diversity by FMT, probiotics or prebiotics decreased the endotoxemia levels and ammonia serum and simultaneously prevented complications and improved prognoses (160). During the past decades, only limited types of antiviral drugs have been successfully developed for a few viruses, such as HIV, HSV and HCV (161). In addition, the emergence of drug-resistant viruses and the need to discover efficient targets for more kinds of viruses remain difficult problems. Under these circumstances, regulating the intestinal microbiota is a promising adjuvant therapy in viral infections. However, there are many limitations in the studies on the mechanism of mutual regulation between viruses and the intestinal microbiota. Studies on human viruses have long been hindered by the lack of a strong culture system and suitable animal models (86). For example, for HuNoV, the interaction between the same virus and the same strains of intestinal microbiota may be quite different *in vivo* and *in vitro* since other factors, such as glycosylation and mucosal immunity in the intestine, can affect viral invasion. As a result, the mechanisms that have been demonstrated in animal models may not occur in humans, and the opposite mechanisms may even occur. The regulation of the intestinal microbiota by specific viruses might be species-specific, and current regulatory methods on the intestinal microbiota in the clinic lack pertinence to some special bacteria, except for common probiotics and pathogenic bacteria and primarily work by enhancing intestinal innate immunity. In most cases, it seems that intervention measures to intestinal microbiota, including FMT or probiotic administration, restore a healthy intestinal community to improve the prognosis of viral infections, lacking discovery of the interrelationship between virions and one or several certain types of bacteria (160). Additionally, regarding FMT, ethical and social issues are present in 5 areas: (1) informed consent and the vulnerability of patients; (2) determining what a 'suitable healthy donor' is; (3) safety and risk; (4) commercialization and potential exploitation of vulnerable patients; and (5) public health implications (146). Although effective supervision measures are necessary, over-restriction also hinders professional care and the development of FMT.

Since the gut microbiota is a very large community that participates in mutual regulation with both the innate and adaptive immune systems of the host, a database of these complex regulations of each single intestinal microbiota type should be developed to regulate specific microbiota.

CONCLUSION

This review summarizes the latest research on the relationship between the intestinal microbiota and viruses as well as the most commonly used methods for regulating the intestinal microbiota, demonstrating that the intestinal microbiota might represent a promising target for antiviral therapy. The regulation of the intestinal microbiota through probiotics and FMT is promising for viral infection therapy. However, the specific mechanisms of the interactions between the intestinal microbiota and viruses require further study, and experimental models that more closely mimic the human internal environment are needed. More evidence is needed to verify the safety and efficacy of FMT, and more targeted regulatory tools must be developed since the effects of the microbiota on viral infection depend on both the individual virus and host.

AUTHOR CONTRIBUTIONS

MY, YY, and MZ drafted the manuscript. YY generated the figures. ML and JX performed the background research. QH, PZ, and MZ edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Contribution of Gut Microbiota to Immunological Changes in Alzheimer's Disease

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Emerging evidence suggests that both central and peripheral immunological processes play an important role in the pathogenesis of Alzheimer's disease (AD), but regulatory mechanisms remain unknown. The gut microbiota and its key metabolites are known to affect neuroinflammation by modulating the activity of peripheral and brain-resident immune cells, yet an overview on how the gut microbiota contribute to immunological alterations in AD is lacking. In this review, we discuss current literature on microbiota composition in AD patients and relevant animal models. Next, we highlight how microbiota and their metabolites may contribute to peripheral and central immunological changes in AD. Finally, we offer a future perspective on the translation of these findings into clinical practice by targeting gut microbiota to modulate inflammation in AD. Since we find that gut microbiota alterations in AD can induce peripheral and central immunological changes *via* the release of microbial metabolites, we propose that modulating their composition may alter ongoing inflammation and could therefore be a promising future strategy to fight progression of AD.

Keywords: Alzheimer's disease, gut microbiota, neuroinflammation, immune cells, therapeutic intervention, microbial metabolites

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder of which the prevalence and disease burden are increasing simultaneously with an aging population (1, 2). Extracellular amyloid-beta (Aβ) deposition and intracellular accumulation of hyperphosphorylated tau are the primary neuropathological hallmarks of AD (3), but increasing attention addresses an additional role of distorted immune responses, although underlying mechanisms remain unknown (4, 5).

Gut microbiota are important for peripheral and central immune homeostasis (6). Species of microbiota and their metabolites can induce peripheral immune activation and contribute to a systemic immune response (7). Moreover, they can modulate integrity of the blood-brain barrier

(BBB) (8), which regulates migration of immune cells into the brain (5). In addition, the production of certain metabolites by gut microbiota is linked to the maturation and function of microglia, the CNS resident immune cells (9).

Over thousands of microbial taxa are present in the adult gastrointestinal tract (GI) where interaction takes place between the host, microbial antigens, and environmental factors (1, 10). Most of these belong to the gram-negative phylum *Bacteroidetes* (1, 11–15) and the gram-positive phylum *Firmicutes* (1, 11–14, 16) with a smaller proportion of gram-positive *Actinobacteria* (11, 14, 17), and gram-negative *Proteobacteria* (14, 18) and *Verruccomicrobia* (12, 19). Within the two dominant phyla, abundant genera include *Bacteroides*, *Clostridium, Faecalibacterium*, *Roseburia* and *Eubacterium* (11, 12, 14). Alterations in microbiota composition occur with increasing age (7), starting around 65 years and include an increase in *Bacteroidetes* and a decrease in *Firmicutes* (20, 21).

Gut microbiota can affect host immunity *via* the release of metabolites and toxins. Microbial misbalance can lead to systemic inflammation in the gut and affect the gut barrier function, increasing permeability and the entry of bacteria, metabolites and toxins into the circulation (22) (**Figure 1**). Interestingly, gut permeability was recently reported to be increased in a cohort of dementia patients together with the occurrence of systemic inflammation (23). Lipopolysaccharide (LPS) is a pro-inflammatory endotoxin found in the outer membrane of gram-negative bacteria like *Bacteroidetes* (15, 24, 25). Besides LPS, some gram-negative species also excrete polysaccharide A (PSA), which has an anti-inflammatory potential (7, 26–28). Other metabolites that predominantly exert immunoregulatory properties are short-chain fatty acids (SCFAs), of which butyrate in particular is known to be produced by species within *Firmicutes*, mainly within clusters of the *Clostridia* class (16, 29–31).

During adult life, microbiota composition can be influenced by different factors such as diet (2, 32, 33), environment (34), body mass index (BMI) (34), cholesterol (34), lifestyle factors such as smoking and exercise (32), drug use (32), and ethnicity (35, 36). As such, microbiota components differ greatly between individuals (37). Despite this variation, a common set of microbial taxa can be found across individuals with diverse dietary habits, geographic origin and ethnicity, referred to as the "core microbiome" (12, 38–40). The core microbiome (11, 12) is essential for microbial functions even if not carried out by the same group of microbes (10) and depends on the expression

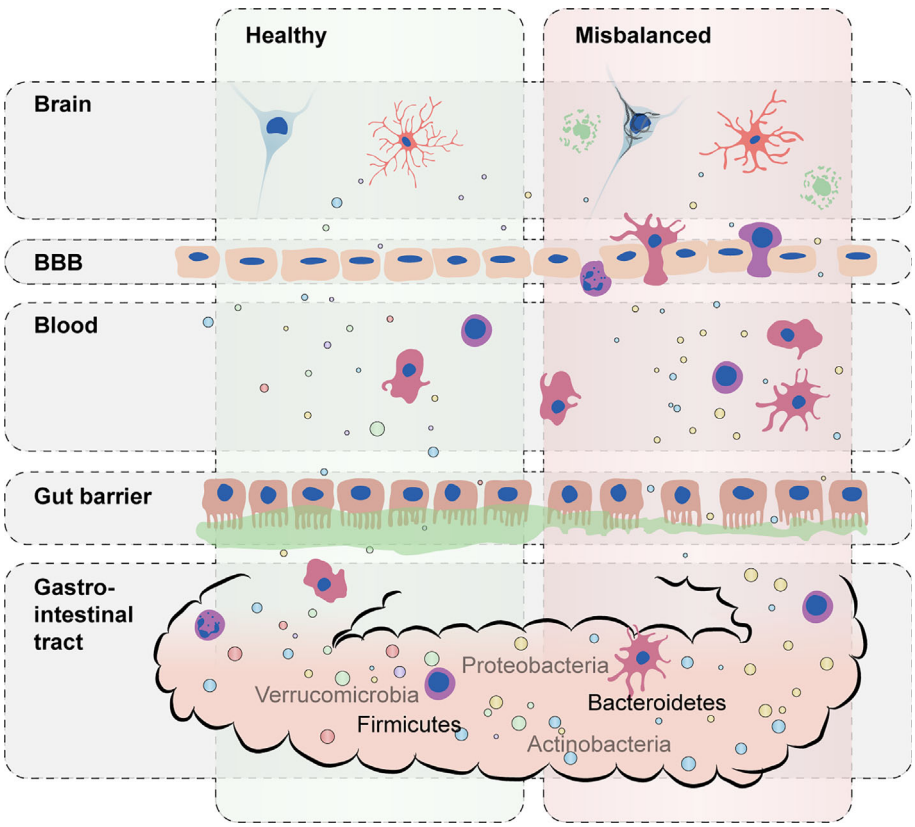

FIGURE 1 | Schematic representation of the impact of a misbalanced gut microbiota on host immunity. A balanced composition of gut microbiota with a high diversity of commensal bacteria carrying out essential microbial functions supports healthy immune responses (left). During microbiota misbalance (right), excessive proinflammatory cytokines and bacterial toxins (e.g., lipopolysaccharide) can lead to disruption of gut permeability and blood–brain barrier (BBB) integrity. Distorted immune responses in the brain can further accelerate and worsen AD-associated pathology such as Aβ and tau accumulation.

of specific combinations of microbial genes, together with metabolic processes and regulatory pathways. Hence, higher diversity in microbiota composition is associated with better health (41).

Aging is the most important risk factor for AD. During aging, the gut microbiota composition decreases in diversity and stability (42). It has been postulated that these changes may evoke hyperstimulation of the immune system resulting in persistent, low-grade inflammation (43), referred to as "inflammaging" (4), a phenomenon observed in the elderly. In addition, decreased immune function, or 'immunosenescence', is a hallmark of aging. Both inflammaging and immunosenescence contribute to aging of the peripheral immune system, which is associated with higher susceptibility to infection, increased risk of autoimmune diseases and impaired cognitive function (4, 43).

In this literature review, we will discuss gut microbiota alterations in both animal model and human studies of AD. Altered microbial taxa and associated metabolites will be described in relation to changes in immune and BBB function. Finally, we will elaborate on possible strategies to target gut microbiota to restore immune homeostasis in AD.

MICROBIOTA COMPOSITION IN AD PATIENTS AND AD ANIMAL MODELS

Animal Studies

Various studies have investigated gut microbiota composition in AD mouse models (**Supplementary Table 1**) (44–55), most in the context of Aβ pathology (44–50, 52–55). Microbial taxa described by three or more studies are shown in **Figure 2**, while **Supplementary Table 2** demonstrates all altered taxa in AD mouse models compared to wild type (WT). All studies observed changes in microbiota composition at one or more taxonomic levels in mouse models of AD compared to WT, although very few alterations were mentioned at species level (only shown in **Supplementary Table 2**).

Studies that investigated changes in microbiota composition during the disease course in animal models found that *Firmicutes* and *Bacteroidetes* abundance both increased and decreased compared to levels in the WT (48, 53, 54). Butyrate producers like the family *Lachnospiraceae* reduced during pathology in AD mice of both sexes while *Roseburia*, a genus within *Lachnospiraceae*, increased over time in male AD mice (54).

Both higher (44, 49, 52) and lower (45, 51) abundance was reported of the phylum *Firmicutes* in AD mouse models. Families and genera that contain butyrate-producing species like the family *Lachnospiraceae* (49, 51, 52) and the genus *Roseburia* (50–52) were also both increased and reduced in AD mice compared to WT. Notably, the butyrate-producing genus *Ruminococcus* was either unchanged or decreased (46, 47, 49). Similar to the abundance of and within *Firmicutes*, higher (45, 51) and lower (44, 49, 52) abundance was reported of the LPS-containing phylum *Bacteroidetes* and of its families and genera (46, 48, 49, 51, 52, 54, 55). Only the genus *Odoribacter* was either unchanged or increased (46, 53).

The different findings regarding microbiome alterations in AD mouse models in the aforementioned studies appear to be independent of age, diet and the type of model, which was either based on Aβ pathology in the APP/PS1 (45–50, 52, 54, 55), 5XFAD (44, 53) and Tg2576 models (54), or on tau pathology in the P301L model (51). Divergent results in microbiota composition can be a result of the use of male or female mice. First, because microbiota composition in male and female mice is under the influence of sex-specific hormones (56, 57) and second, because AD pathology manifest itself differently between sexes. Studies using only male mice (44, 49, 52) showed a reduction in *Bacteroidetes* and an increase in *Firmicutes* in AD mouse models, while studies using both males and females varied in their results (45, 51, 53). At each age, the ratio of *Firmicutes/Bacteroidetes* remained lower in AD than in WT mice in females (48), suggesting that females more often show an increase in *Bacteroidetes* and a reduction in *Firmicutes* as opposite to male AD mice. Gram-negative families such as *Bacteroidaceae* are both increased and reduced in male mice in relation to AD (46, 49, 52, 55), but more often increased in females (48, 54). Families that encompass butyrate-producers like *Lachnospiraceae* (49, 52) show increase in male mice, while studies that included females or both sexes demonstrated a decrease (51, 54). Microbiota alterations in the gram-negative genus *Bacteroides* and butyrate-producing genus *Roseburia* across different studies seem less sex-specific, although one study demonstrated an increase in *Bacteroides* in females specifically, while males show an increase in *Roseburia* (54). Since the large majority of studies use either male mice or a combination of males and females, it is possible that the overall results are more specific for males than they are for females.

Altogether, it seems that various alterations in microbiota composition are associated with pathology and disease progression in AD animal models. This suggests that general microbiota misbalance, rather than alterations in specific taxa, is characteristic for AD.

Human Studies

So far, only three studies have directly compared gut microbiota composition in AD patients to controls (58–60) and one studied microbiome associations with amyloid pathology (61) (**Supplementary Table 3**).

Both an increase (58) and a slight decrease (59) in abundance of the phylum *Bacteroidetes* was reported in AD patients (**Figure 3**). All species within the phylum *Bacteroidetes* are gram-negative and contain the toxin LPS in their outer membrane (15, 24, 25). Higher circulating levels of LPS were associated with increased Aβ deposition in elderly patients with cognitive complaints (62). Increased abundance was detected in most genera within *Bacteroidetes* and most of its species, including *B. fragilis* (60), which produces polysaccharide A (PSA) (7). Interestingly, *B. fragilis* was decreased in cognitively impaired patients that were Aβ-positive compared to healthy subjects (61).

Abundance of the phyla *Firmicutes* was both reduced (58) and unchanged (59) in AD patients. Within *Firmicutes*, a reduction was seen in the family *Lachnospiraceae* (59, 60) the genus *Roseburia* (60) and the genus *Eubacterium* (60), which all

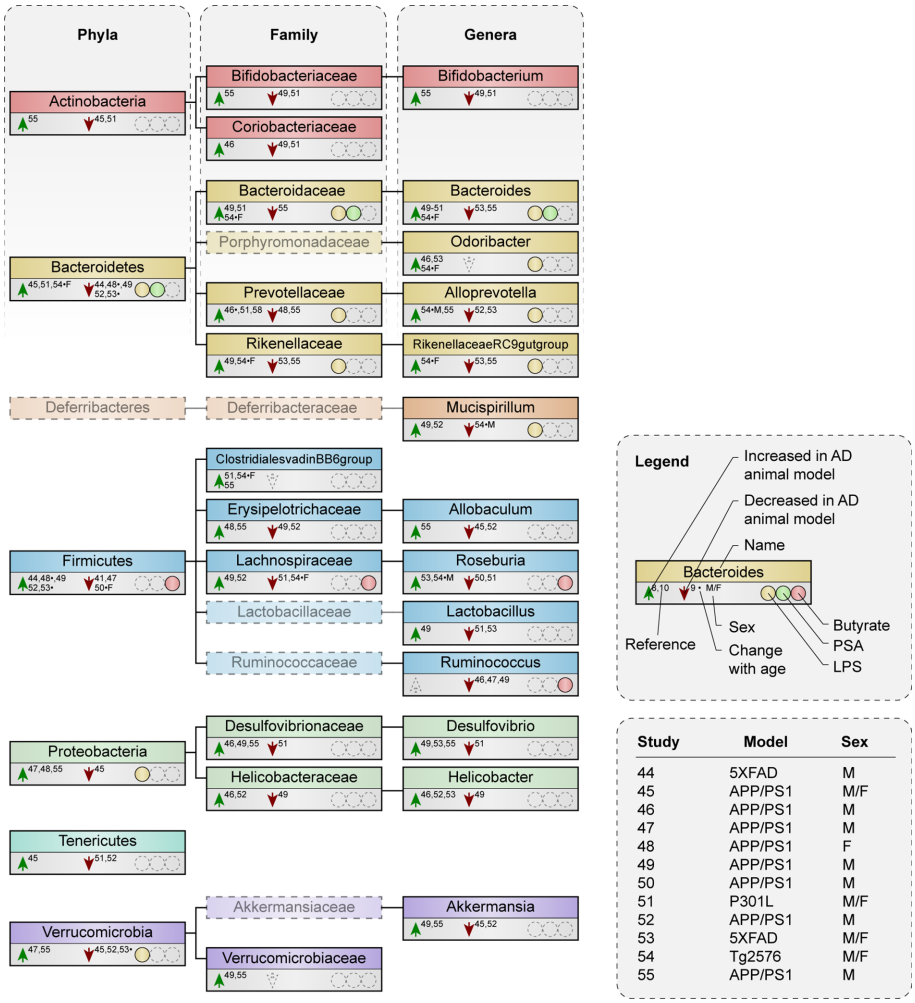

FIGURE 2 | Microbial taxa are altered in AD mouse models compared to WT mice. Phylogenetic representation at phylum, family and genus level of microbial taxa described by three or more animal studies. Animal studies are represented that either compare microbiota composition between AD and WT mice at certain age point(s), or that examine alterations with increasing age in AD mice compared to WT. Arrows indicate an increase or decrease in abundance of a certain taxa in AD mouse models compared to WT. * indicates a result was observed in AD mice with increasing age, but not in WT. F or M show a change that was only seen in females or males respectively, if both were included in one study. Presence or excretion of toxin lipopolysaccharide (LPS; yellow) and metabolites polysaccharide A (PSA; green) and butyrate (red) is indicated, as well as the used animal model and sex of the animals used per study.

harbor species that produce the metabolite butyrate. At species level, butyrate-producers like *R. hominis*, *F. prausnitzii, E. rectale* and *E. hallii* were lower abundant in AD patients (60). Interestingly, butyrate levels in plasma negatively associated with Aβ deposition in cognitively impaired patients (62).

The deposition of Aβ in cognitively impaired patients positively associates with the abundance of the gram-negative genus *Escherichia/Shigella*, while the butyrate-producing species *E. rectale* negatively relates to Aβ deposition in these patients, both as compared to Aβ-negative cognitively impaired patients and healthy controls (61). In addition, differences in variation of microbial taxa within an individual, which is also called α-diversity and associates with better health (41), was decreased in AD patients (58).

Available evidence regarding microbiota composition in patients with AD faces several limitations, including small sample sizes and limited data on and adjustment for dietary intake and other relevant confounding factors, such as co-morbidity, use of medication and lifestyle. Only two of the aforementioned studies (60, 61) reported microbiota composition at species level. Few studies related the observed differences between groups to disease biomarkers and severity, such as cerebral spinal fluid (CSF) or PET biomarkers for Aβ and tau, MRI characteristics or cognitive functioning. Therefore, results should be interpreted with caution. Limited human data points towards higher abundance of gram-negative species containing LPS while species that produce the metabolite butyrate were decreased. Besides, phyla, families and genera

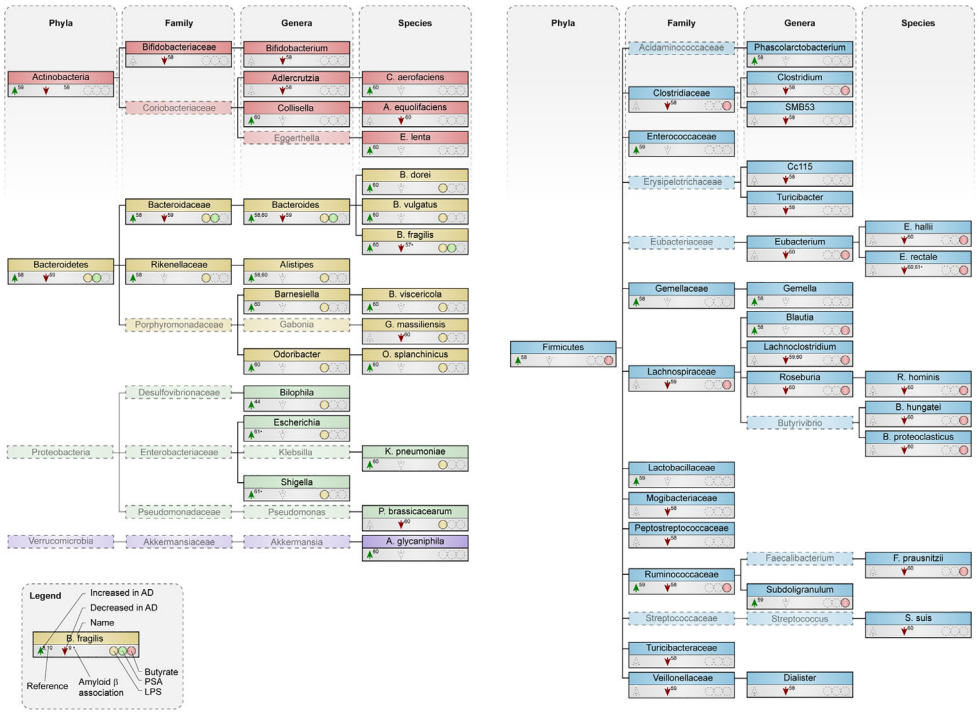

FIGURE 3 | Microbial taxa are altered in AD patients compared to control subjects. Phylogenetic representation at phylum, family, genus and species level of microbial taxa in human studies. Arrows indicate an increase or decrease in abundance of a certain taxa in AD patients compared to healthy controls. * depict changes associated with amyloid pathology in cognitively impaired patients. Presence or excretion of toxin lipopolysaccharide (LPS; yellow) and metabolites polysaccharide A (PSA; green) and butyrate (red) is indicated.

encompassing these LPS-containing and butyrate-producing species were also mostly increased and decreased respectively.

MICROBIOME-IMMUNE INTERACTIONS

Bacteroidetes and *Firmicutes* make up the largest portion of the adult microbiota and showed most changes in abundance in AD patients and in relevant animal models. Here, we discuss how species of *Bacteroidetes* and *Firmicutes* and their metabolites cause activation or inhibition of peripheral and central immune cells and how they affect function of the BBB (**Figure 4**).

Gram-Negative Bacteria

Bacteria within the phylum *Bacteroidetes* are gram-negative and contain LPS (15, 24, 25). LPS can induce systemic inflammation *via* Toll-like receptor (TLR)-4 signaling (15, 24, 25) and promotes the secretion of proinflammatory cytokines like interleukin 1 and 6 (IL-1 and IL-6) and tumor necrosis factor α (TNF-α) (63). IL-1 and IL-6 are required for differentiation of T-helper 17 (Th17) cells (64), which *via* proinflammatory cytokine release and their action on neurons *via* the Fas/Fas-ligand apoptotic pathway, are thought to contribute to neuroinflammation and neurodegeneration in AD (65). In addition, LPS of the species *B. fragilis* induced signaling *via* nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in human neuronal-glial co-cultures, an important pathway in inflammatory neurodegeneration (66). Moreover, increased abundance of the genus *Bacteroides* positively associated with cerebrospinal fluid (CSF) levels of chitinase 3 like protein 1 or YKL-40, which is a marker for microglial and astroglial cell activation (58). Increases in species of *Bacteroidetes* might contribute to LPS transport from the intestines to the brain, adding to AD pathology (33, 67). Interestingly, LPS has been found at higher levels in the parenchyma and vessels of AD brains compared to aged-matched controls, and co-localized with Aβ plaques around blood vessels (67).

LPS has also been described to increase P-glycoprotein (P-gp) expression at the intestinal epithelial barrier (68) and the BBB (69) but to reduce its activity (68, 69). P-gp is a protein highly expressed at the brain endothelium (70, 71), where it functions as an efflux transporter, and is involved in the clearance of Aβ across the BBB (70–72). In AD, P-gp expression and function at the BBB is decreased (66, 73–75), contributing to Aβ accumulation in the brain (74, 75). Interestingly, fecal supernatants isolated from AD patients decreased expression of P-gp in an intestinal epithelial monolayer culture, compared to supernatants from control subjects and elders with other dementia types (60). The expression of P-gp was influenced by the abundance of several gram-negative *Bacteroides* species. High

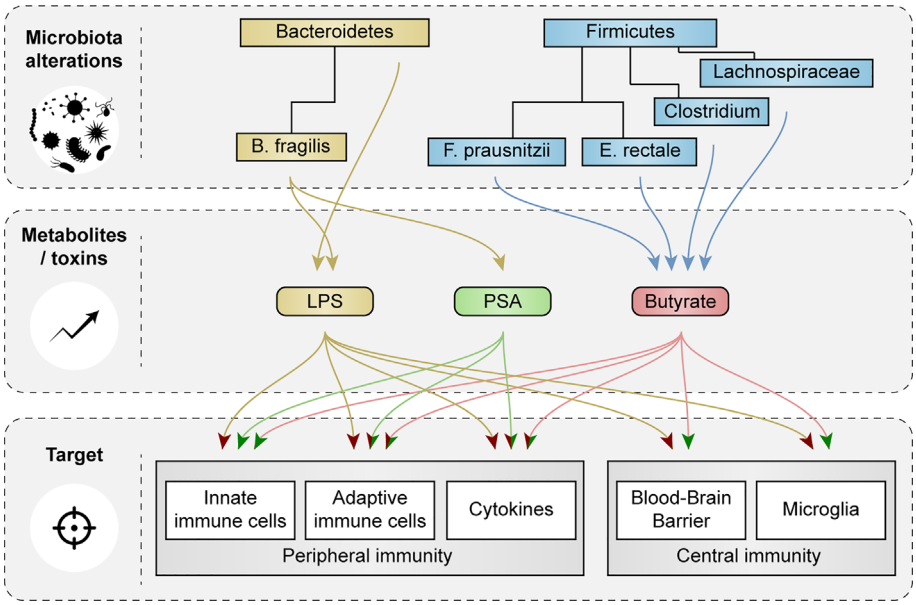

FIGURE 4 | Schematic representation of the effects of microbial metabolites and toxins on peripheral and central immunity and blood-brain barrier function. Impact of microbial metabolites and toxins, that were changed in AD patients and in relevant animal models, on peripheral and central immune cells, cytokine secretion and blood-brain barrier (BBB) function are showed. Red arrow heads indicate a pro-inflammatory effect or loss of BBB integrity, green arrow heads indicate tolerogenic effects and improvements in BBB function.

levels of the species *B. dorei* increased P-gp expression while increased abundance of *B. fragilis* and *B. vulgatus* correlated to decreased expression of P-gp (60).

In addition, the presence of meso-diaminopimelic acid (meso-DAP) in the peptidoglycan layer of the bacterial cell wall, which is part of all gram-negative and some gram-positive bacteria, is recognized by the nucleotide-binding oligomerization domain-containing protein 1 (NOD1) (76). Excretion or translocation of gram-negative peptidoglycan increased activity of bone marrow-derived neutrophils *via* NOD1 receptor signaling (77).

Gram-Negative Bacteria: PSA Producers

Polysaccharide A (PSA) is a capsular carbohydrate that specifically derives from the gram-negative species *B. fragilis*. PSA from *B. fragilis* promotes regulatory immune responses *via* binding to TLR-2 (26–28), including the induction of dendritic cells (DCs) and regulatory T cells (Treg) (26–28) and the secretion of IL-10 (28, 78), together with subsequent inhibition of Th17 cells (26, 27) and suppression of IL-17 production (79). Colonization of germ-free (GF) mice with PSA-producing *B. fragilis* led to an expansion of $CD4^+$ T cell levels in the spleen and restored the Th1/Th2 cytokine balance of GF mice by reducing IL-4 production and restoring interferon γ (IFN-γ) expression (80). Interestingly, another study that investigated PSA-mediated stimulation of $CD4^+$ T cells found that cells responding to PSA displayed an unusual combination of pro-inflammatory cytokines (IFN-γ, TNF-α, IL-6 and C-X-C motif chemokine ligand 10) and anti-inflammatory surface receptor expression (Lag3, Tim3, Pd1) that was mainly driven *via* the interferon signaling pathway (81). Hence, the immunological response to PSA exposure is highly context dependent and cannot be considered simply regulatory or pro-inflammatory.

Butyrate-Producing Bacteria

Short-chain fatty acids (SCFAs) promote gut integrity and play an important role in both physiological and pathological conditions (82–84). The most abundant SCFAs are acetate, butyrate and propionate, of which butyrate is particularly essential in the gut, as it is the most important metabolic substrate required for colonocyte proliferation and differentiation (82, 85). Butyrate-producing bacteria are widely distributed amongst the gram-positive phylum *Firmicutes* (16). Two of the most important butyrate-producing species are *F. prausnitzii* and *E. rectale* (29, 86), which exert different effects on peripheral immunity, including the induction of Treg cells (87, 88) and inhibition of NF-κB signaling in the intestinal epithelium (89). Additional butyrate producing taxa can be found within other bacterial families, mostly in *Lachnospiraceae* (see **Figures 2** and **3**) (90–98)

Butyrate, like other SCFAs, exerts its effects by acting as an histone deacetylase (HDAC) inhibitor (99) and *via* G-protein receptor (GPCR) signaling (29), which can both lead to inhibition of the NF-κB signaling pathway (100–103). Butyrate-mediated inhibition of HDAC signaling downregulated inflammatory mediators IL-6, IL-12 and nitric oxide synthase 2 (NOS2) in LPS-treated macrophages *in vivo*, and *in vitro* (31). Furthermore, butyrate-induced GPCR signaling increased IL-10 expression in splenic dendritic cells (DCs) and macrophages *in vitro* (30) and

enhanced plasma IL-10 levels *in vivo* (104). DCs and macrophages cultured with butyrate had increased potency to induce differentiation of Treg cells (30, 104–107) and promoted IL-10 production by $CD4^+$ T cells, while decreasing levels of IL-17 (30). Butyrate-induced Treg differentiation *in vitro* was dependent on transforming growth factor β1 (TGF-β1) (107). Notably, butyrate administration in rats reduced IL-6, IL-17 and IL-23 levels and increased levels of TGF-β in the plasma (104). DCs exposed to butyrate could also suppress differentiation of naïve T cells into pro-inflammatory IFN-γ producing T cells (105). Butyrate also reduced the production of TNF-α and cytokine-induced neutrophil chemoattractant (CINC) 2αβ and nitric oxide (NO) in LPS-treated rat neutrophils *in vitro* (99).

However, a high dose of butyrate induced IFN-γ and T-bet expression, both associated with a Th1 phenotype, in $CD4^+$ T cells cultured under Treg cell inducing conditions (107). Unlike the induction of Treg cells, induction of these Th1 associated factors was not dependent on TGF-β1. Under Th17-polarizing conditions, butyrate inhibited RORγt and IL-17A but induced IFN-γ, while under Th2-polarizing conditions, butyrate decreased expression of GATA3 and IL-4 and induced IFN-γ. The upregulation of IFN-γ under these conditions is dependent on the expression of T-bet. In addition, butyrate upregulated IFN-γ in a concentration dependent manner in unpolarized T cells (107). As such, it has been proposed that butyrate might exert its pro-inflammatory potential in an inflammatory context, while it shows anti-inflammatory effects under homeostatic conditions (107). Still, most evidence points to butyrate as a potent anti-inflammatory SCFA both *in vivo* and *in vitro*.

IMMUNE-AD ASSOCIATIONS

While AD pathology was long considered to be driven mainly by Aβ and tau pathology, accumulating evidence shows that dysfunctional neuro-immunological responses considerably contribute to AD pathogenesis and might even be a driving factor (5). Rare genetic variants associated with AD are often highly expressed in microglia, including the triggering receptor expressed on myeloid cells 2 (TREM2) (108). Rare variants of TREM2 are associated with a two- to threefold increase in risk of AD development (109). Besides, the role of the immune system in AD is not limited to the brain, but also involves peripheral immune signaling (4, 5, 110). Accordingly, blood-derived leukocytes were identified in the brain of AD patients and AD animal models (111–113), and infiltration of these peripheral immune cells into the brain during disease pathogenesis can be facilitated by an increase in BBB inflammation and enhanced permeability (114).

Through the release of cytokines, complement proteins and major histocompatibility complex (MHC) class I proteins, peripheral immunity can affect CNS homeostasis (115). Besides, factors that exert their functions in the CNS, like neurotransmitters, are involved in the mediation of immune responses through corresponding receptors on innate and adaptive immune cells (116–118). In addition, the CNS can control systemic immune responses *via* the vagus nerve (119), and the sympathetic branch of the autonomic nervous system can influence intestinal immunity and homeostasis (120). Activation of the HPA axis and subsequent release of glucocorticoids also greatly affects immune responses (121). The recently discovered brain lymphatic system (122–124) and the regulation of immune cell trafficking across the BBB (125) further contribute to communication between the peripheral immune system and the CNS (125). As discussed, microbiota can exert different effects on central and peripheral immunity *via* their metabolites and toxins, thereby affecting central and peripheral inflammatory processes in AD.

THERAPEUTIC STRATEGIES TARGETING THE GUT MICROBIOTA AND METABOLITES IN AD

Pro-, Pre- and Antibiotics

Probiotics

Probiotics consist of living microbes and can introduce beneficial microbial components that are missing in the host (126). In APP/PS1 mice, treatment with probiotics containing *Bifidobacterium longum* and *Lactobacillus acidophilus* in combination with exercise was able to inhibit the progression of cognitive impairment and Aβ deposition (50). These species, among other strains within *Bifidobacterium* and *Lactobacillus*, provide cross-feeding to butyrate-producers (127). Before treatment, APP/PS1 mice showed higher abundance of several *Bacteroides* species and a reduction of butyrate-producing strains compared to WT mice. Probiotic treatment in combination with exercise decreased the gram-negative species *B. fragilis* and *Bacteroides thetaiotaomicron*, of which the latter was related to poorer spatial memory, while both of these species were increased by probiotics alone. Butyrate-producing genera like *Eubacterium* and *Roseburia* were enhanced by probiotic treatment in combination with exercise, but decreased by probiotic treatment alone. Exercise without probiotic supplementation was also able to reverse the alterations in butyrate producing species and in *B. fragilis* that were seen in APP/PS1 mice, but did not decrease *B. thetaiotaomicron*. Accordingly, spatial memory was improved by exercise and probiotic treatment combined, but not considerably altered by exercise or probiotics separately. Aβ pathology was decreased by probiotics and exercise separately, and by combined treatment (50).

AD patients who received probiotics containing species of *Bifidobacterium bifidum*, *Lactobacillus fermentum*, *Lactobacillus casei* and *Lactobacillus acidophilus*, which provide cross-feeding to butyrate-producing bacteria, showed improvement on the Mini Mental State Exam (MMSE) compared to untreated patients (128). Probiotic treatment in AD patients also resulted in favorable changes in insulin metabolism and in malondialdehyde (MDA) and high sensitivity C-reactive protein (hs-CRP), which are markers for oxidative stress and inflammation respectively, but was ineffective on other biomarkers of oxidative stress and inflammation such as total antioxidant capacity (TAC), nitric oxide (NO) and glutathione (GSH). Of note, the effect of probiotic treatment on microbiota composition was not investigated and as such, no firm

conclusions can be drawn whether results were mediated by probiotic-induced changes in microbiota composition (128).

Prebiotics

Prebiotics are defined as substrates selectively used by host microorganisms to produce health benefits. The main source of prebiotics are plant-derived carbohydrate compounds called oligosaccharides (129). Prebiotics are non-digestible by the host, selectively fermented by intestinal microorganisms and selectively targeting and stimulating growth and activity of beneficial bacteria, especially *Bifidobacterium* and, to a lesser extent, *Lactobacillus* (129). Rats that received a hippocampal injection of Aβ42 and were orally treated with oligosaccharides from *Morinda officinalis* (OMO) afterwards show improved learning and memory in a dose dependent way, ameliorated neuronal loss, decreased Aβ42 expression and reduced oxidative stress. In these rats, OMO treatment decreased both pro-inflammatory cytokines and anti-inflammatory IL-10 to a level similar to WT. Moreover, OMO treatment restored the abundance of both *Bacteroidetes* and *Firmicutes* to WT levels (130). In APP/PS1 mice, OMO treatment induced an increase in *Firmicutes*, particularly in the butyrate-producing family *Lachnospiraceae*, while a decrease was seen in *Bacteroidetes* and the genus *Bacteroides*. Interestingly, an increase in *Firmicutes* and *Lachnospiraceae* and a reduction in *Bacteroidetes* were also observed in APP/PS1 mice compared to WT. Similar to rats, OMO treatment improved learning and memory in a dose dependent way (49). Together, these studies show that OMO treatment can affect different aspects of AD pathology like neuronal loss, cognitive deficits, inflammation, oxidative stress and Aβ42 expression, and that OMO might exert these effects *via* modulating microbiota composition (49, 130).

Antibiotics

Antibiotics are commonly used to limit bacterial colonization of the body, without targeting specific taxa, and can lead to significant alterations in gut microbiota composition (131).

Studies have reported that antibiotic treatment can ameliorate neuroinflammation and other aspects of AD pathology, including Aβ and tau accumulation and oxidative stress (132). Antibiotic treatment in AD mouse models affected AD pathology in a sex-specific manner. Male APP/PS1 mice showed a significant decrease in Aβ plaque compared to untreated animals after antibiotic treatment, a result that was not observed in females (133). In males, antibiotic treatment reduced microglial and astroglial reactivity around Aβ plaques (133), while an activated microglial phenotype was observed independent of antibiotic treatment in female mice (134). Additionally, antibiotic treated males had decreased expression of pro-inflammatory cytokines like IL-1β and IL-17A, while these cytokines were increased in antibiotic treated females. Furthermore, the antibiotic treatment induced sex-specific changes in microbiota composition. Also, antibiotics inhibited pathways related to LPS synthesis, but this effect was stronger in males than in females (134). These results were at least partially microbiome-dependent since microbiota transplantation from untreated male APP/PS1 mice to antibiotic treated mice resulted in partial restoration of Aβ deposition and microglial morphology (134).

So far, contradicting results have been reported between clinical trials. One study demonstrated that high doses (50-100 mg) of the antibiotic D-cycloserine, administered over a period of 4 weeks, improved cognition in AD patients (135), while an earlier study that treated patients with lower doses (15 mg) for the same period showed no effects (136). In 2004, a combined treatment of doxycycline and rifampicin for a period of 3 months resulted in significantly less cognitive decline over the 6-month period after the start of the treatment in patients with probable AD and mild to moderate dementia, compared to the placebo treated group (137). In contrast, a later study in 2013 found no beneficial effects of a 12-month treatment with either doxycycline or rifampin, or combined treatment, on cognition in AD patients (138).

The reason for these discrepancies regarding the effects of antibiotics might be the multifactorial nature of AD, or other systemic effects of the antibiotic besides changing microbiota composition. The outcome of clinical trials can also be affected by participants being infected with *H. pylori*, which is quite common in older patients. As such, cognitive improvement that is observed in infected patients might be a result of elimination of the *H. pylori* infection by antibiotics (131, 139). Also, while some studies examined the effect of antibiotic treatment directly after the treatment period (135, 136, 138), one performed cognitive assessment months after the treatment had stopped (137). In both cases, the treatment was reported to improve cognitive decline (135, 137), suggesting cognition was improved by a significant reduction in microbial diversity or in specific taxa right after antibiotic treatment, but also by a changed composition after repopulation. Still, other studies showed no effect of antibiotics directly after the treatment (136, 138).

In summary, research suggests that the use of antibiotics can at least interfere with AD pathology and associated neuroinflammation. However, microbiome-independent effects of antibiotics in GF mice, which lack microbiota, have been described that included changed host metabolites and inhibited respiratory activity in immune cells, consequently impairing immune phagocytic activity (140). In addition, antibiotic treatment has shown to induce FoxP3$^+$ Treg cell in GF animals (141). Hence, it can be debated if the reported neuro-protective effects of antibiotic treatment were mediated *via* changes in the gut microbiome or *via* other pathways. Future research should elucidate if the effects of antibiotic treatment are mediated *via* changes in microbiota composition, *via* direct effects on immune cells or *via* other pathways in the host.

Polysaccharide A (PSA) Treatment

No research has yet been performed on the possible beneficial effects of PSA on cognition or neuroinflammation in AD. However, PSA treatment reduced disease severity of mice with experimental autoimmune encephalomyelitis (EAE), which is often used to model the neuro-inflammatory disease multiple sclerosis (MS) (142). In addition, treatment of EAE mice with PSA reduced neuroinflammation by reducing pro-inflammatory cytokines in a TLR2 dependent manner (143) and through inhibition of Th1 and Th17 responses (142). If PSA administration can interfere with pathological processes in AD remains to be investigated. However,

since the immunological response to PSA exposure could be dependent on the inflammatory context (81), other therapeutic strategies targeting the gut microbiota and metabolites in AD might be more promising for future research.

Butyrate Treatment

Treatment with butyrate in GF male mice decreased BBB permeability and increased expression of the tight junction protein occludin. The same effect on BBB permeability was observed after monocolonization of GF mice with the butyrate producing bacteria *Clostridium tyrobutyricum* (144). Besides, pretreatment of adult and aged mice with a single injection of butyrate decreased LPS-induced IL-1β expression in microglia. Notably, the observed effect was stronger in aged mice. Pretreatment with butyrate also decreased LPS-induced IL-1β expression in the hippocampus of aged mice (145).

In APP/PS1 mice studies at advanced disease stage, treatment with butyrate resulted in improved memory function *via* HDAC inhibition, but did not affect Aβ pathology (146). Accordingly, treatment of aged Tg2576 mice with butyrate also improved cognition without affecting Aβ pathology, but decreased tau pathology and improved synaptic plasticity (147). In 5XFAD mice, butyrate both decreased Aβ deposition and improved cognition. Here, the effect of the treatment in an early disease stage was examined (148), suggesting that the effect of butyrate on Aβ deposition might be dependent on disease stage. Additionally, treatment of APP/PS1 mice with *Clostridium butyricum* increased fecal butyrate concentrations and ameliorated cognitive deficits and neurodegeneration, suppressed microglia activation and decreased levels of the pro-inflammatory cytokines Il-1β and TNF-α. Besides, *Clostridium butyricum* reversed microbiota alterations that were observed in APP/PS1 mice (149). No studies have yet been performed on the effect of butyrate treatment on AD pathology in humans. Altogether, usage of butyrate both reversed microbiota alterations and was able to interfere with neuroinflammation, BBB permeability, cognitive decline and, in early stage, pathological hallmarks like Aβ and tau in mouse models of AD.

Additional Dietary Interventions

Calorie Restriction

The effects of dietary restriction, which can be either caloric reduction or intermittent fasting, on neuroinflammation are well summarized by Bok et al., which states that dietary restriction can reduce neuroinflammation *via* several mechanisms, including inhibition of the NF-κB pathway, or attenuation of aged-associated pro-inflammatory activation of astrocytes and microglia (150). Calorie restriction (CR) rescued most microbiota alterations that occur with increasing age in Tg2576 AD mice, and downregulated genes associated with intestinal inflammation (54) and reduced Aβ pathology (151). In contrast, CR also upregulated transcription factor Rorγt, which promotes a Th17 response (54). Interestingly, the CR diet as described by Cox et al. restricts only in carbohydrates (54), which are a source for butyrate production (16). However, if CR lowered butyrate levels was not investigated.

High Fiber Diet

Dietary intervention with fibers has shown to affect gut microbiota composition and levels of SCFAs. Supplementation of diets of healthy young adults for 2 weeks with three fermentable fibers resulted in an increase in SCFAs, including butyrate. As a response to resistant starch from potatoes, some participants showed an increase in *Ruminococcus bromii* or *Clostridium chartatabidum*, and this was associated with higher butyrate concentrations, especially in the presence of *E. rectale* (152). High fiber diet decreased the expression of inflammatory cytokines like IL-1β, TNF and IL-6 in microglia from adult and aged mice (145). Expression of these cytokines was negatively correlated to cecal levels of butyrate (145). Hence, a high fiber diet is able to modulate neuro-immunological processes probably *via* an increase in butyrate levels.

Mediterranean Diet

Adherence to a Mediterranean diet (MD) is associated with a lower risk for developing AD and delay in cognitive decline (153). This diet is characterized by high intake of fruits, vegetables, legumes, nuts, cereals, olive oil and fish, moderate intake of dairy, low intake of meat, and small quantities of wine (154). Through its antioxidant properties, MD is beneficial in combating oxidative stress in AD (153). Besides, dietary components like beneficial unsaturated fatty acids provide anti-inflammatory actions (155), and MD was found to be associated with lower levels of inflammatory markers like C-reactive protein (CRP) and IL-6 (156). MD also affects the gut microbiota and its metabolites, and has been linked to increased production of SCFAs (157). A cohort study with 153 Italian individuals following different diets showed that adherence to the MD correlates to higher fecal levels of acetate, propionate and butyrate (158). Accordingly, MD has been associated with increased abundance of butyrate-producing strains like *F. prausnitzii* (157, 159) and *E. rectale* (160) and the butyrate-producing genus *Roseburia* (161) and to reduced levels of circulating LPS (162).

Fecal Microbiota Transplantation

Fecal microbiota transplantation (FMT) is the infusion of feces from a healthy donor into the gut of a recipient with the aim of targeting microbiota composition and is a promising strategy for combating disease associated with microbiota imbalance (163–168). FMT treatment is generally considered safe, especially after extensive donor screening and testing (169, 170) but remains technically challenging (donor selection and preparation of the fecal transplant). In the future, specific supplementation of a (combination of) beneficial strains could be more feasible on a larger scale (169).

In APP/PS1 mice, FMT from WT mice for a period of 4 weeks improved spatial memory and reduced Aβ accumulation and tau phosphorylation. Besides, FMT increased expression of proteins involved in synaptic plasticity, PSD-95 and synapsin I, and decreased inflammatory protein Cox-2 in the cortex and hippocampus. In addition, FMT reduced CD11b expression, which is a marker for microglia and other myeloid cells (171, 172), that was increased in APP/PS1 compared to WT mice.

Alterations in microbiota composition in APP/PS1 mice compared to WT, including a reduction in *Bacteroidetes*, were reversed by FMT. Moreover, FMT increased levels of butyrate (171). Another study shows that FMT from WT to ADLPAPT mice, a relatively newly developed AD mouse model, resulted in decreased formation of Aβ plaques and neurofibrillary tangles, reduced glial activity and improved cognition (173). So far, no studies have investigated the effect of FMT in AD patients. However, an effect of FMT on the brain was demonstrated in obese patients, where FMT increased dopamine transporter binding which was associated with an concomitant increase in *Bacteroides uniformis* (174).

Overall, it seems that FMT treatment in AD mouse models has the potential to reverse microbiota alterations, improve cognition and synaptic plasticity, decrease Aβ and tau pathology and to reduce neuroinflammation (171, 173).

DISCUSSION

This review shows different alterations in microbiota composition in AD. Compositional differences across AD mouse studies were contradicting, as gram-negative and butyrate-producing bacteria were both increased and decreased in abundance. Limited but available human data revealed a higher abundance of gram-negative species within *Bacteroidetes* while species within *Firmicutes* that produce the metabolite butyrate were decreased. As such, it seems that results observed in mouse models of AD are not completely translatable towards humans.

Differences in microbiota composition between AD patients and AD animal models might be explained by differences in anatomy (175, 176) or in the used research techniques; while human studies use stool samples, cecal contents are mostly used in mouse studies (176). Another possible explanation is the absence of correction for confounding factors such as age, sex, diet, comorbidities, use of medication and inclusion of small sample sizes in human studies. Moreover, discrepancy in the findings might also be a result of the male/female ratio in these studies as sex-differences in gut microbiota composition have been acknowledged in humans (177) and in mice (56, 57, 178, 179). In AD mouse models, disease manifestation differs between males and females. Rapid and more severe AD pathology has been reported in females compared to males, including increased Aβ pathology (180, 181), tau phosphorylation (180) and neuroinflammation (181, 182) and cognitive deficits (183). Possibly, different disease manifestation between sexes in AD transgenic mouse models influences microbiota composition or vice versa. In humans, variation in AD disease manifestation has also been observed between men and women, and includes differences in cognitive symptoms and brain atrophy (184). Further research should shed more light on the possible relationship between sex-differences in disease manifestation and variations in microbiota composition in AD.

Lastly, all but one mouse study used a transgenic AD mouse-model with mutations associated with familial AD leading to early and excessive Aβ pathology. As such, detected shifts in microbiota composition are probably more characteristic to Aβ pathology of familial AD and can differ when other AD-associated pathologies are investigated. Also, mice and humans standardly show differences in microbiota composition, and raising the animals under specific pathogen free (SPF) conditions can reduce microbial diversity. Differences between mice and humans in microbiome research might be closed by using mice with humanized microbiota (185). However, generation of such animals is difficult, as successful transplantation of human gut microbiota into mice is under influence of anatomical and physiological factors, diet and environmental stimuli (186).

Innate immune activation in AD might differently affect pathogenesis dependent on disease stage. An activated microglial response could limit Aβ pathology in early or middle stages of AD (187–189), while it exacerbates tau pathology and neuronal loss in late stages (189, 190). Since the effects of immune activation on AD pathogenesis seem to be dependent on disease stage, the relation between the gut microbiome and AD could be as well. Hypothesizing, inflammatory effects of the gut microbiota might inhibit aspects of AD pathology in early disease stages, while they exacerbate pathology in late stages. The variation in microbiota composition in AD observed between different studies in humans (58–60) and animal models (44–55) might also be dependent on disease stage. Hence, it would be interesting to investigate functional changes in microbiota composition over the course of disease progression in humans together with shifts in immune status. Accordingly, animal studies show microbiota alterations in AD mouse models over time (48, 53, 54), suggesting that microbial changes correspond with disease progression.

Another possible way in which gut microbiota can induce immune activation is *via* their release of bacterial amyloids. Bacteria produce amyloids as part of their biofilm, a self-produced extracellular matrix which protects the population from different environmental and host insults (191). Although bacterial-derived amyloids differ in structure from CNS amyloid, it has been suggested that due to existing similarities in their tertiary structure (192, 193), bacterial amyloids may prime the immune system and induce misfolding of other host proteins through molecular mimicry (191, 194–197). For example, bacterial amyloid curli is recognized by the TLR2/TLR1 complex that also recognizes human Aβ. Apart from *Escherichia coli* and *Salmonella enterica serovar Typhimurium*, most of these observations are made *in vitro* (191). Hence, future research should reveal *in vivo* interactions between bacterial amyloid, the immune system and AD pathology.

Treatment strategies that target the microbiota and their metabolites, including pro-, pre- and antibiotics, butyrate treatment, and dietary interventions, have shown potential to modulate neuroinflammation and/or improve other aspects of AD pathology (**Figure 5** and **Supplementary Table 4**). Prebiotics and dietary interventions such as a high fiber might be able to restore the functional core of the microbiome by providing necessary dietary compounds for beneficial microbial metabolism. Less is known about butyrate-treatment or FMT as a therapeutic option for AD. However, both treatments were able to restore microbiota imbalance, enhance cognition and synaptic plasticity, decrease AD pathology and to reduce neuroinflammation in mouse

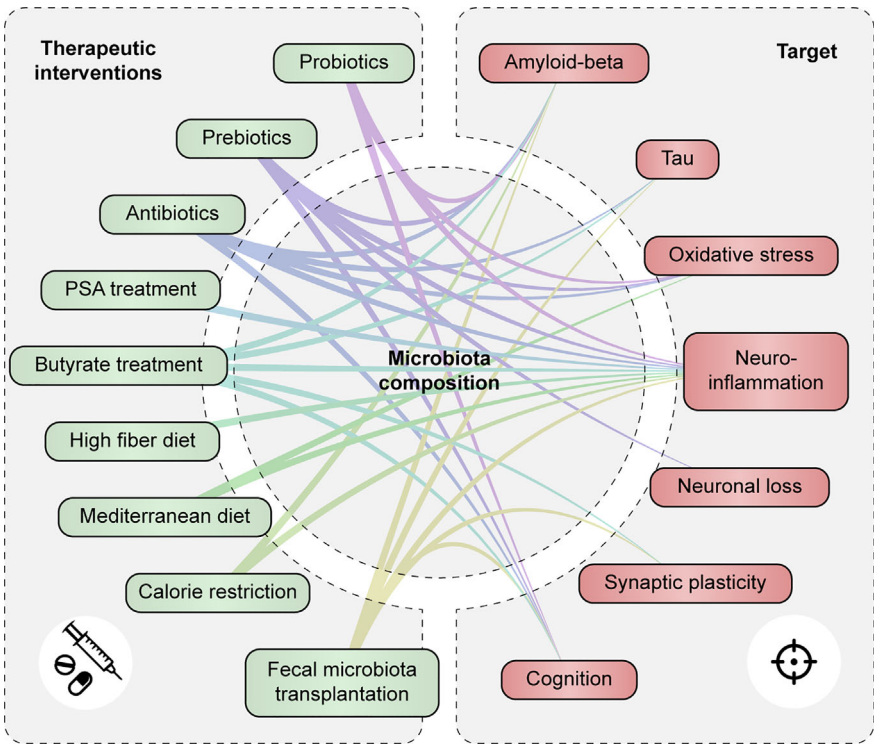

FIGURE 5 | Therapeutic strategies targeting the gut microbiota and metabolites modulate AD-associated pathology. Associations are shown between therapeutic interventions that modulate gut microbiota composition and/or function and AD-associated pathologies such as tau and Aβ accumulation, oxidative stress and neuroinflammation, neuronal loss, synaptic plasticity and cognitive function.

models of AD. As such, these therapeutic options could be promising to follow up in future studies.

In conclusion, gut microbiota composition shows many changes in AD patients and animal models, despite some inconsistencies in compositional differences between studies. It is clear however, that species of bacteria affect central and peripheral immune networks and have the ability to modulate ongoing neuro-immunological responses. Thus, restoring misbalanced microbiota in AD may present a future measure to increase immune fitness and alleviate AD pathology.

Still, recovering microbial balance with no general consensus on what characterizes the AD-associated microbiome is doomed to face long odds. To overcome inconsistencies between studies in this field, future research should move towards studying the functionality and dynamics of the human core microbiome in AD rather than static abundance of microbial taxa, for example, by studying levels of immunomodulating metabolites, like PSA or butyrate, in relation to AD. Also, a better understanding is needed of how such dynamic alterations affect immune pathways and how these pathways can be therapeutically targeted. A good starting point would be the use of shotgun sequencing (38, 198, 199) accompanied by immune profiling at different time-points of disease. Larger sample sizes in clinically well characterized cohorts could enable assessment of confounding/mediating effects of sex and other host factors such as diet in the relation between microbiota function, immune status and AD pathology. Finally, future studies should address how the microbiota-associated changes relate to AD biomarkers and disease severity, such as cerebral spinal fluid (CSF) or PET biomarkers for Aβ and tau, MRI characteristics or psychological assessment of cognitive functioning.

AUTHOR CONTRIBUTIONS

LO and SR wrote the manuscript. AK designed the figures. BV, AG, and MM provided valuable scientific input and revised the manuscript. LO, WF, and HV conceived the study and were involved in the overall supervision and editing of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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MINI REVIEW
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First Encounters: Effects of the Microbiota on Neonatal Brain Development

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The microbiota plays important roles in host metabolism and immunity, and its disruption affects adult brain physiology and behavior. Although such findings have been attributed to altered neurodevelopment, few studies have actually examined microbiota effects on the developing brain. This review focuses on developmental effects of the earliest exposure to microbes. At birth, the mammalian fetus enters a world teeming with microbes which colonize all body sites in contact with the environment. Bacteria reach the gut within a few hours of birth and cause a measurable response in the intestinal epithelium. In adults, the gut microbiota signals to the brain via the vagus nerve, bacterial metabolites, hormones, and immune signaling, and work in perinatal rodents is beginning to elucidate which of these signaling pathways herald the very first encounter with gut microbes in the neonate. Neural effects of the microbiota during the first few days of life include changes in neuronal cell death, microglia, and brain cytokine levels. In addition to these effects of direct exposure of the newborn to microbes, accumulating evidence points to a role for the maternal microbiota in affecting brain development via bacterial molecules and metabolites while the offspring is still *in utero*. Hence, perturbations to microbial exposure perinatally, such as through C-section delivery or antibiotic treatment, alter microbiota colonization and may have long-term neural consequences. The perinatal period is critical for brain development and a close look at microbiota effects during this time promises to reveal the earliest, most primary effects of the microbiota on neurodevelopment.

Keywords: neonatal, microbiota, human, mouse, neural, cell death, microglia, maternal

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INTRODUCTION

The womb has long been assumed to be sterile, with the first direct exposure to microbes occurring at birth. Recently, this concept has been questioned, with reports of a microbial signature in the placenta, amniotic fluid, and fetal gut (e.g., Rackaityte et al., 2020). However, other studies do not see offspring-associated microbiota in healthy pregnancies distinct from contaminating DNA, hence supporting the "sterile womb" hypothesis (e.g., de Goffau et al., 2019; reviewed in Walter and Hornef, 2021). Regardless of this debate's outcome, all agree that microbes from maternal and environmental sources rapidly and densely colonize the neonate at birth.

Although microbes colonize all body surfaces in contact with the environment, over 98% of our body's microbes are located within the gastrointestinal tract. The gut microbiota has effects on the brain and behavior in adulthood, as demonstrated most directly by studying animals with absent or a reduced microbiota throughout life. Germ-free (GF) mice, for example, have alterations in social behavior, stress responding, cognition, and other functions (Cryan and Dinan, 2012). Some of these effects can be corrected with exposure to a microbiota in adolescence, but others persist (e.g., Clarke et al., 2013), suggesting that early life is a sensitive period for effects of microbe exposure on the brain.

In this review, we focus on microbe exposure in the immediate peri-partum period to address key questions and identify gaps in our knowledge related to: (1) how microbes signal to the neonatal brain, (2) which microbes first colonize the gut and exactly when that occurs, (3) what the effects are of microbe exposure on the neonatal brain, and (4) whether effects of microbe exposure on brain development begin *in utero*.

WHAT PATHWAYS SIGNAL THE ARRIVAL OF THE FIRST MICROBES?

Although the gut microbiota is comprised of bacteria, viruses, fungi, and protozoa, bacteria have received by far the most attention to date. In adults, gut bacteria communicate with the brain in at least four ways: via direct neural connections, bacterial metabolites, hormones, and immune signaling. Much less is known about gut-brain signaling in neonates (**Figure 1**), but we have at least a rudimentary understanding of what pathways are operational during this period.

The Vagus Nerve Establishes Connections With the Gut Prenatally

The vagus nerve, which innervates the intestines from the proximal duodenum to the distal descending colon, is a major bidirectional communication system between the gut and brain. Primary sensory neurons of the vagus reside in the nodose ganglion and send a peripheral projection to the gut wall and a central projection to the nucleus of the solitary tract (NTS) in the hindbrain. The NTS, in turn, projects to several forebrain areas, such as the paraventricular nucleus of the hypothalamus (PVN) and arcuate nucleus, conveying messages related to the chemical contents of the gut, intestinal distension and inflammation, gut hormone release, and other information (Browning et al., 2017; Fülling et al., 2019). In mice, vagal sensory fibers innervate the duodenum by embryonic day (E) 14 and the distal small intestine by E16 (Ratcliffe et al., 2011). Thus, vagal connections are in place to convey the earliest information from gut microbiota to the brain. It has yet to be demonstrated, however, whether bacterial signals from the gut lumen signal to the brain via the vagus nerve in the first hours or days after birth.

Vagal innervation of the gastrointestinal tract promotes the proliferation of enteroendocrine cells (EECs), which are specialized cells in the intestinal epithelium capable of sensing the presence of microbes and relaying that information to the brain (Buchanan and Bohórquez, 2018). A subset of EECs termed neuropod cells are electrically excitable and directly synapse with vagal nerve endings. Neuropod cells can relay information from the gut to the nodose ganglion in milliseconds, which is faster than any previously known mechanism (Kaelberer et al., 2018). Although EECs are present on the day of birth in newborn rodents (Penkova et al., 2010), more work is needed to understand whether the neuropod cell-to-vagus connection is present and functional at this age.

Microbial Metabolites Signal to the Brain

Hundreds of bacterial metabolites penetrate host body tissues (Uchimura et al., 2018), and some of these, such as aryl hydrocarbon receptor ligands, short-chain-fatty acids (SCFAs), tryptophan, and secondary bile acids have demonstrable effects on the host (e.g., Arpaia et al., 2013; Gomez de Agüero et al., 2016). SCFAs (e.g., acetate, propionate, and butyrate) have been especially well studied for their role in the gut-brain connection and are produced by bacteria as end products of the fermentation of indigestible dietary fibers. SCFAs can be shuttled across the gut epithelium by monocarboxylate transporters and act locally on the vagus nerve (Silva et al., 2020). They also can cross the blood brain barrier (BBB) where they may bind to free fatty acid receptors expressed in the brain or act via epigenetic mechanisms (Berni Canani et al., 2012; Falomir-Lockhart et al., 2019). Although SCFAs can be measured in circulation in perinatal mice (Kimura et al., 2020), and monocarboxylate transporters are expressed by capillary endothelial cells forming the BBB of newborn rodents (Omori et al., 2020), it has yet to be directly demonstrated whether SCFA signaling occurs in the newborn brain.

Gut Hormones

Gut peptides, such as cholecystokinin, peptide YY, gastric inhibitory peptide, and ghrelin are produced by EECs and other gastrointestinal cells. The microbiota regulates the levels of these gut peptide hormones, which activate receptors on vagal afferents in the intestinal mucosa (Lach et al., 2018) or in the brain to affect neural circuits controlling eating behavior (Andermann and Lowell, 2017). Gut peptide hormones are measurable prenatally and approach adult levels by birth (Bryant et al., 1982); whether the release of these hormones is affected by the arrival of the pioneer microbiota in newborns is another area ripe for investigation.

Immune Signaling

Immune signaling is a fourth route of microbiota-gut-brain signaling. For example, the innate immune receptors toll-like receptor (TLR) 4 and TLR5 are activated by the bacterial antigens lipopolysaccharide and flagellin, respectively. Both receptors are expressed at high levels and are fully functional in gut epithelial cells of perinatal mice (Gribar et al., 2009; Fulde et al., 2018). TLR5 selects against flagellated bacteria during the neonatal period (Fulde et al., 2018) and TLR4 binding in epithelial cells causes the release of proinflammatory cytokines (Gribar et al., 2009). In adults, this cytokine release activates brain regions such as the PVN (Rivest, 2001). Assuming that cytokines circulating in

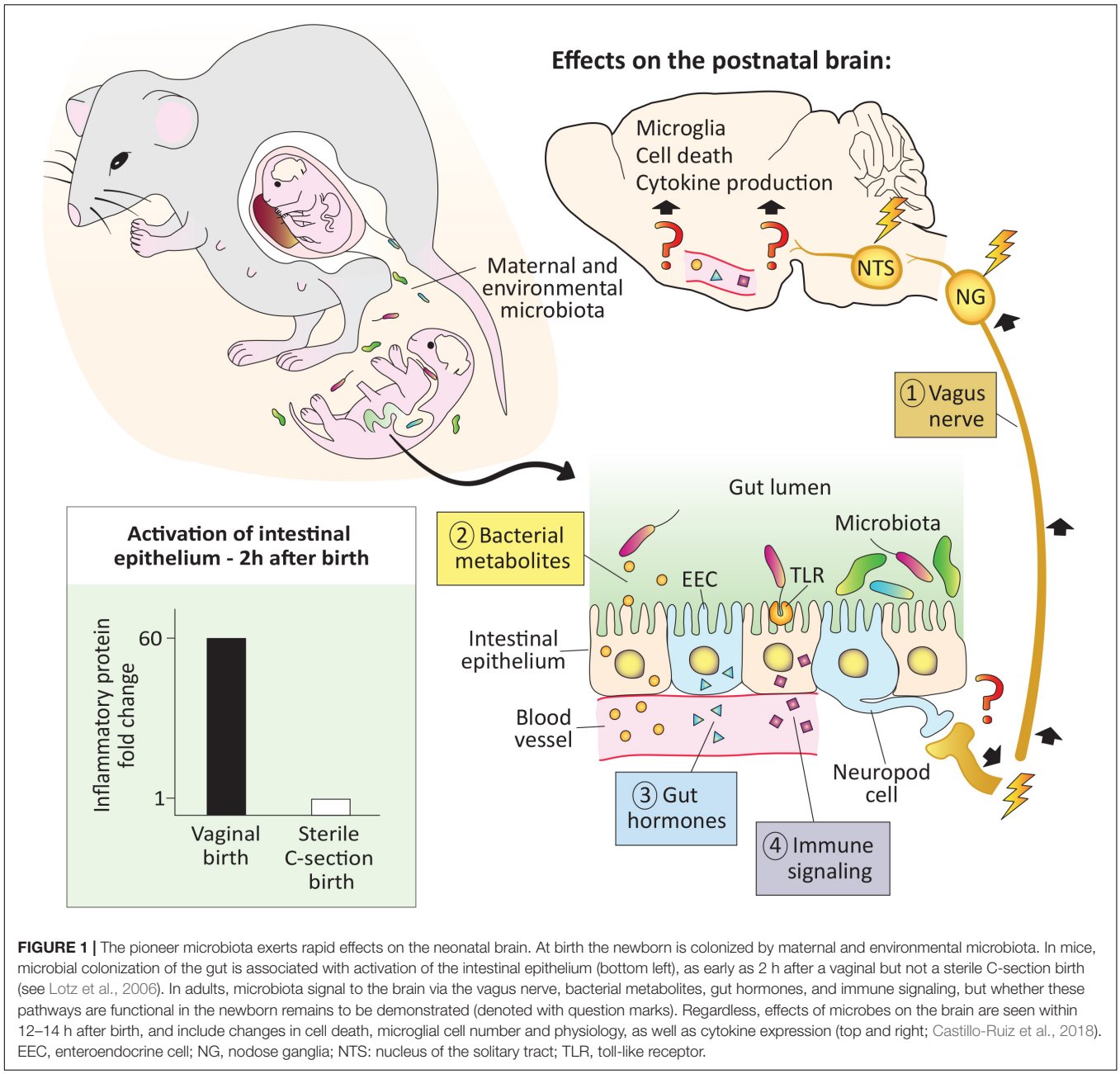

FIGURE 1 | The pioneer microbiota exerts rapid effects on the neonatal brain. At birth the newborn is colonized by maternal and environmental microbiota. In mice, microbial colonization of the gut is associated with activation of the intestinal epithelium (bottom left), as early as 2 h after a vaginal but not a sterile C-section birth (see Lotz et al., 2006). In adults, microbiota signal to the brain via the vagus nerve, bacterial metabolites, gut hormones, and immune signaling, but whether these pathways are functional in the newborn remains to be demonstrated (denoted with question marks). Regardless, effects of microbes on the brain are seen within 12–14 h after birth, and include changes in cell death, microglial cell number and physiology, as well as cytokine expression (top and right; Castillo-Ruiz et al., 2018). EEC, enteroendocrine cell; NG, nodose ganglia; NTS: nucleus of the solitary tract; TLR, toll-like receptor.

neonates also signal to the brain, this system may be in place at birth to detect the earliest-arriving microbes. In addition, TLRs are expressed in the perinatal mouse brain (e.g., Kaul et al., 2012), which may allow for direct brain sensing of bacteria-related molecules, although we currently know very little about their regional distribution in the perinatal brain.

THE PIONEER MICROBIOTA – WHEN DO THEY ARRIVE AND "WHO" ARE THEY?

The newborn begins its lifelong exposure to gut microbes with its first swallow. Low levels of bacteria are detected in the gastric aspirate of human babies within 1 h of a vaginal but not a C-section birth, suggesting that these bacteria are acquired during passage through the birth canal (Bajorek et al., 2019). Almost immediately after birth the baby begins feeding, leading to the ingestion of almost one million bacteria daily from breast milk (Le Doare et al., 2018), although how bacteria get into the milk remains controversial (Greer et al., 2019). Microbes are present in human meconium samples on the first postnatal day (Hansen et al., 2015), and there is already evidence of a nascent microbiome in the lower intestinal tract of mice within a few hours of birth (van Best et al., 2020).

One might reasonably question whether the tiny numbers of microbes present in the first hours of life have any meaningful

effect on the host, but two observations suggest that they may. In the Hawaiian bobtail squid, the development of the light-emitting organ is dependent on a specific bacterial species (*Vibrio fischeri*) entering the organ during a sensitive period. Remarkably, the bacteria populate the organ within minutes of hatching, and as few as 5 individual bacteria are sufficient to trigger its development (Altura et al., 2013). In mammals, the earliest functional effects of postnatal microbes reported to date may be the microbe-dependent activation of intestinal epithelial cells that is seen just 2 h after birth in mice (Lotz et al., 2006). This activation resolves several hours later, as the epithelial cells achieve tolerance. Interestingly, tolerance does not occur in mice born by sterile C-section and isolated from the dam (Lotz et al., 2006), demonstrating the requirement for direct exposure to microbes for this important process. This work also speaks to the "sterile womb" hypothesis: if bacteria are present in the fetal intestine, they are not numerous enough to trigger the activation of the intestinal epithelium that occurs just 2 h after exiting the womb.

Human and mouse studies have sought to characterize the pioneering gut bacteria in the first days of life. Pantoja-Feliciano et al. (2013) report that the earliest colonization of the mouse gut begins with the presence of *Streptococcus* 1 day after birth, followed by dominance of *Lactobacillus* (responsible for fermenting milk lactose) by day 3. The neonate transitions to a more stable gut microbial community dominated by *Bacteroides* around the time of weaning. This work largely replicates the findings made over 45 years ago by Schaedler (1973) who used culture techniques to characterize the pioneer microbiota in mice. Human studies have classified a similar transition in the first days of life from aerobic species (including *Streptococcus*) to a more diverse microbial profile including bacteria with varying oxygen requirements, including anaerobic or facultative anaerobic species (e.g., *Lactobacillus*), though this is then followed by dominance of *Bifidobacterium* by 1 week of life (Fanaro et al., 2003).

Thus, gut microbes are present within the first few hours of life, and a succession of dominating genera takes place in neonatal mice and humans. Moreover, these bacteria, even in very small numbers, can have an effect on the periphery. In the last 2–3 years, effects of these pioneer microbes on brain development have also been reported.

HOW DOES THE PIONEER MICROBIOTA IMPACT BRAIN DEVELOPMENT?

The microbiota colonizes the newborn's body during a time when the brain is being shaped by key developmental processes. In mice, these include colonization of the brain by microglia and developmental neuronal cell death. Microglia, the resident immune cells of the brain, increase markedly in number, and change in morphology and gene expression during the early postnatal period in mice (Nikodemova et al., 2015). Similarly, cell death, which eliminates roughly 50% of post-mitotic neurons via apoptosis, is concentrated during the first postnatal week in mice (Ahern et al., 2013). Neuronal cell death shows abrupt changes following birth (Mosley et al., 2017) which led us to hypothesize that microbiota colonization at birth may play a role in shaping this process.

Using GF mice, we found that microbiota absence at birth is associated with region-specific changes in cell death and increased microglial labeling in the hippocampus and hypothalamus (Castillo-Ruiz et al., 2018; **Figure 1**). These effects were not seen prenatally, but occurred within 12–14 h of birth, suggesting that direct exposure to microbes is necessary. We also found that the expression of pro-inflammatory cytokines, especially interleukin 1β and tumor necrosis factor α, was markedly higher in the brains of mice born in the presence of a microbiota than in those born GF (Castillo-Ruiz et al., 2018). More recently, we reported neural activation 3 h after birth in the PVN (Hoffiz et al., 2021), a brain region that receives input from the vagus nerve and has a central role in immune regulation and the stress response. This timing coincides with the arrival of microbiota to the newborn gut, although we have yet to demonstrate that the two events are causally linked. Since oral gavage with pathogenic bacteria causes activation of the PVN within 2 h in adults (Wang et al., 2002), it is certainly possible that pioneer bacteria could signal to the PVN very rapidly postpartum, and this is an important area for future study.

Other neurodevelopmental processes may be affected by microbiota colonization at birth in mice and in other species. The stage of brain development at birth varies between short- and long-gestation species, so the neural processes affected by the first exposure to a microbiota are also likely to vary. In humans, for example, cell death is ongoing at birth, but microglial colonization occurs prenatally (Menassa and Gomez-Nicola, 2018).

DO EFFECTS OF THE MICROBIOTA BEGIN *IN UTERO*?

Regardless of the final outcome of the "sterile womb" debate, evidence is strong that microbes play an essential role in fetal development, including brain development, via *indirect* effects through the mother (**Figure 2**).

Maternal Bacterial Metabolites Reach the Fetus

In mammals, all calories and nutrients required for fetal growth are transferred from the mother, and the microbiota influences this transfer in several ways. For example, the maternal gut microbiota increases the energy harvested from food, and is essential for the synthesis of vitamins and the generation of SCFAs (Macpherson et al., 2017). Recent evidence demonstrates that molecules derived from the maternal microbiota reach the fetus to influence gene expression and anatomical development of the brain.

In a global metabolomic analysis, Li et al. (2020) found that roughly 5% of the metabolites present in human fetal intestine and meconium could be classified as "microbial" (i.e., metabolites that are either produced by microbes or

produced by the host in response to microbes). Because they did not detect any microbial signature in the fetus itself, the authors hypothesize that the microbial metabolites are vertically transmitted from the mother. Similarly, molecules from isotope-labeled bacteria administered to pregnant mouse dams reach the placenta and fetal circulation (Gomez de Agüero et al., 2016). In addition, when GF dams are transiently colonized with a genetically engineered strain of *E. coli* that does not persist in the intestine passed term, their offspring have more innate leukocytes and altered intestinal gene expression compared to pups born to unmanipulated GF dams (Gomez de Agüero et al., 2016). Some of the changes in the offspring of transiently colonized dams are related to the expression of antibacterial peptides and mucus production, and pups of gestation-only colonized mothers are better adapted to microbial challenges later in life. Thus, indirect effects of the maternal microbiota may prepare the fetus for the direct exposure to microbes encountered at birth.

Maternal Microbiota-Dependent Metabolites Affect Brain and Peripheral Nervous System Development of Offspring

Fetuses gestating in GF dams have reduced expression of endothelial tight junction proteins and increased BBB permeability compared to fetuses of conventional control dams (Braniste et al., 2014), indicating that the maternal microbiota influences *in utero* development of the BBB. The microglia in male mouse embryos of GF mothers are more numerous and highly branched, and show substantial changes in gene expression compared to those of controls (Thion et al., 2018), demonstrating that development of the brain's innate immune system is also affected by maternal microbial status.

Mouse embryos from antibiotic-treated and GF dams exhibit reduced thalamo-cortical axon growth (Vuong et al., 2020). This abnormality is due to a reduction in maternal microbiota-dependent metabolites reaching the fetus, and is prevented by colonizing the dams with even a limited set of bacteria (Vuong et al., 2020). Development of the peripheral nervous system is also affected by maternal microbial metabolites that reach the offspring *in utero*. For example, radioactively labeled SCFAs administered to the maternal colon reach their embryos within 40 min, and embryos lacking specific free fatty acid receptors have reduced development of sympathetic nerve projections to the heart (Kimura et al., 2020).

Thus, microbial molecules and microbe-dependent metabolites from the mother affect offspring neural development *in utero* (**Figure 2**). Koren et al. (2012) and others have reported dramatic changes in the maternal gut microbiota over the course of pregnancy, which suggests that the metabolites reaching the offspring may also vary during gestation. However, other studies report relatively stable microbial communities over pregnancy (e.g., Yang et al., 2020). The reason for the discrepancy is unclear, but could include differences in subject-related factors across studies, such as maternal age and body weight.

COMMON PERTURBATIONS OF THE MICROBIOTA THAT MAY AFFECT OFFSPRING BRAIN DEVELOPMENT

Although humans are never born GF (but see: Barnes et al., 1969), many variables of modern life can profoundly alter the maternal

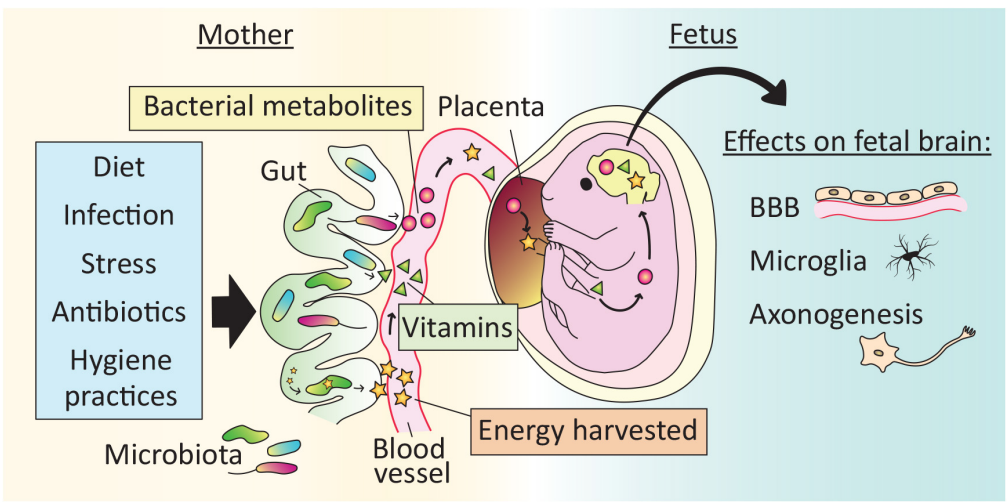

FIGURE 2 | Effects of the microbiota on brain development begin *in utero*. The maternal gut microbiota exerts effects on fetal brain development indirectly via the production of vitamins and bacterial metabolites, as well as by increasing the energy harvested from food. Microbe-dependent molecules cross the placenta and may reach the fetal brain. Reported effects of the maternal microbiota on fetal brain development include development of the blood brain barrier (BBB), microglial cell number and physiology, and axonogenesis. As a result, perturbations to the maternal microbiota, for example via lifestyle (e.g., diet), illness (e.g., stress or infection) or medical treatment (e.g., antibiotics), may affect offspring brain development.

and newborn microbiota (**Figure 2**). Birth mode, birth timing, maternal infection, antibiotic exposure, breast milk vs. formula feeding, hygiene practices, maternal diet, and maternal stress all cause changes in the maternal and/or offspring microbiota and, in some cases, have been linked to behavioral or brain alterations in the offspring (Cryan et al., 2019; Jašarević and Bale, 2019).

For example, the offspring of female mice fed a high-fat diet before and during pregnancy have social deficits that are attributable to alterations in their microbiota (Buffington et al., 2016). Maternal infection during pregnancy influences offspring cortical development and later behavior in mice, and these effects appear to be mediated via the maternal microbiota (Kim et al., 2017). A staggering 40% of all United States women (and essentially 100% of those undergoing C-section) are now treated with antibiotics immediately prior to delivery (Kuperman and Koren, 2016), with demonstrated profound and surprisingly long-lasting effects on the gut microbiota of the newborn. In boys, neonatal antibiotic treatment impairs growth during at least the first 6 years of life, whereas treatment later in infancy does not (Uzan-Yulzari et al., 2021). Although this latter study was not designed to examine neurological outcomes, restricted childhood growth has previously been associated with poor neurodevelopment (Castanys-Muñoz et al., 2017). These findings, combined with the recent data showing effects of the very early microbiota on normal offspring brain development (section "How Does The Pioneer Microbiota Impact Brain Development?"), suggest that we should be cautious about clinical manipulations that alter the microbiota of newborns. In cases where such manipulations are unavoidable (e.g., antibiotic treatment of newborns with proven infections), knowledge of what microbes support normal neonatal brain development – and when – may allow us to intervene with therapeutic benefit.

WHAT'S NEXT?

It should not come as a surprise that microbes affect the developing brain, since at no time in evolutionary history has a nervous system developed in the absence of signals from microbes. Thus, the mammalian brain may have evolved to be "microbe expectant" in the same way that sensory systems are activity dependent, and require afferent input for normal development. If so, then both the presence and the relative absence of microbes can be potent signals, and interesting hypotheses are suggested for future study. We wonder, for example, whether the "leakiness" of the BBB seen in GF mice (e.g., Braniste et al., 2014) might be due to a feedback mechanism attempting to increase the availability of the (missing) signals from microbes.

Although the effects of the gut microbiota on brain development have been the subject of several published reviews, a careful reading of the literature reveals that few studies have actually examined the developing brain. Instead, effects of microbes on neurodevelopment are largely inferred from studies examining brain chemistry or behavior in adulthood. Observations in adults have been important in establishing long-term, functional consequences of the microbiota on the brain and behavior, but shed little light on exactly how the microbiota influences the developing brain. It is unknown, for example, which brain changes reflect primary responses to the microbiota and which are secondary effects of altered development elsewhere in the nervous system. Our ability to identify how the microbiota affects brain development will be hampered if we ignore the earliest, most direct effects, and the recent studies we have highlighted here are finally addressing this.

Many gaps in our understanding remain, however, including the pathways by which the very earliest microbes signal to the brain. Recent advances in metabolomics, in particular, promise to soon provide a more comprehensive picture of microbial metabolites that mediate effects of the maternal or newborn microbiota on brain development. Research in the gut-brain-axis field is progressing very rapidly, and is likely to soon illuminate this and other questions.

AUTHOR CONTRIBUTIONS

AG, NMR, AC-R, and NGF conceived, wrote, and edited the manuscript. BC provided useful insights and edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Getting on in Old Age: How the Gut Microbiota Interferes With Brain Innate Immunity

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The immune system is crucial for defending against various invaders, such as pathogens, cancer cells or misfolded proteins. With increasing age, the diminishing immune response, known as immunosenescence, becomes evident. Concomitantly, some diseases like infections, autoimmune diseases, chronic inflammatory diseases and cancer, accumulate with age. Different cell types are part of the innate immunity response and produce soluble factors, cytokines, chemokines, and type I interferons. Improper maturation of innate immune cells or their dysfunction have been linked to numerous age-related diseases. In parallel to the occurrence of the many functional facets of the immune response, a symbiotic microbiota had been acquired. For the relevant and situation-dependent function of the immune system the microbiome plays an essential role because it fine-tunes the immune system and its responses during life. Nevertheless, how the age-related alterations in the microbiota are reflected in the innate immune system, is still poorly understood. With this review, we provide an up-to-date overview on our present understanding of the gut microbiota effects on innate immunity, with a particular emphasis on aging-associated changes in the gut microbiota and the implications for the brain innate immune response.

Keywords: gut micobiota, microglia, inflammaging, senescence, metabolites, bacteria, brain, innate immunity

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INTRODUCTION

One of biology's fascinations is the inevitability of death. However, human longevity has never been at the focal point as recently with the advances in socioeconomics, life sciences and medicine. With the increasing proportion of older individuals, comes the burden of maintaining a healthy lifespan. Aging affects multiple organ systems and processes, and increases the risk for neurodegenerative diseases like Alzheimer disease (AD) and Parkinson disease (PD) (Hou et al., 2019). Alterations in immune system functions and defense mechanisms is well described in aged individuals (Dorshkind et al., 2009). Therefore, the ability to delay or reverse the effects of aging on the immune system would have significant advantages. The cardinal features of the immune system are categorized into innate and adaptive functions.

A key difference between innate and adaptive immune cells can be seen when it comes to antigen recognition. The main reason is that the different cell types most likely had to fulfill specific tasks to fight cell death, tumorgenesis or infections. The different cell types responsible for the innate immune response comprise dendritic cells (DCs), macrophages, basophils, neutrophils, natural killer (NK) cells, eosinophils, and monocytes. Although it was long believed that the innate immune cells like macrophages and DCs show a rather "broad-spectrum" mode of recognition, they detect microbes with the help of pattern recognition receptors (PRRs) (Janeway, 1989). As

an example, lipopolysaccharides (LPS) are part of the bacterial cell wall and can be sensed by Toll-like receptor 4 (Park and Lee, 2013). In the case of tissue damages or even cell death as a consequence of trauma or infection, so called "danger associated molecular patterns" (DAMPs) are recognized by other receptor-types. Among others, these molecular structures are presented by uric acid, high mobility group box 1 protein (HMGB1), heat shock proteins (Kono and Rock, 2008), and tumor cell DNA, which activates the stimulator of interferon genes (STING) pathway (Ohkuri et al., 2014; Woo et al., 2014). In addition, the expression of regulatory receptors on NK cells is modulated by other target cells and more specifically their expression level of the major histocompatibility complex (MHC) (Yokoyama, 2005). The primary innate immune cells of the brain are microglia. Microglia resembles tissue-resident macrophages and protect the brain by their reaction to potentially dangerous signals for the CNS cells, they remove cellular debris by phagocytosis, produce various types of cytokines to influence the microenvironment and can contribute to neuronal survival (Nayak et al., 2014). Importantly, innate cell receptors are germline-encoded and heritable, which is not the case for the antigen receptors of adaptive immunity. In this way, cells of the innate immune system are in the first line of defense when it comes to infections or tissue injury.

Multifaceted bacterial communities that colonize different body sites, represent an evolutionarily adjusted ecosystem, which holds an enormous multiplicity of genes that provoke direct interactions with physiological functions (Bischoff, 2016). One of the critical implications is most likely the regulation of the host immune system efficiency. When we look at the human digestive tract, the estimated total number of microorganisms in the intestine equals about the estimated total number of human somatic cells in the human body (Sender et al., 2016). Bacteria make up the highest number of all gut microbiota with about 500–1,000 different bacterial species (Sommer and Backhed, 2013). Bacteroidetes and Firmicutes are the most common bacterial phyla and make up about 90% of the whole intestinal microbiota (Qin et al., 2010). The remnant contains numerous species of other phyla. Although these species are only present in lower number, they might still produce paramount metabolites for salubrious aging. The progression of aging involves an imbalance in the status of the immune system (Nikolich-Žugich, 2018), where the microbiota might be playing a principal role in fine-tuning pro-inflammatory and anti-inflammatory activities of the immune cells (Zheng et al., 2020).

Various reports have described the interaction of the microbiota with brain function during the aging process but the centerpiece of the current review will be the modulation of the innate immune compartment by microbiota.

INNATE IMMUNITY AND BRAIN AGING

Microglia

Accounting for ∼15% of brain cellularity, the most critical innate immune cell for maintaining normal brain function is microglia (**Figure 1**). Microglia constantly monitor their environment with their cell processes while the soma remains rather stationary. While microglia mainly use their cellular processes for monitoring, they can also use them to establish cell-cell contact to neighboring cells and blood vessels (Nimmerjahn et al., 2005). In the cortex of aged individuals, microglial processes are changing in morphology and display a more fragmented and spheroid phenotype (Streit et al., 2004). These morphological changes can indicate cell activation. However, additional research results suggest that the perturbed appearance of microglia during the aging process is rather indicative of dystrophic and senescent cells (Sheng et al., 1998; Miller and Streit, 2007). The age-associated shortening of microglial cell processes and the downregulation of the microglial sensome genes cause a reduced coverage of brain parenchyma. It would be interesting to determine whether microglial age-related changes in morphology are responsible for a reduced cell-cell interaction of microglia with neurons and astrocytes (Hickman et al., 2013). Besides morphological changes of microglia during aging, they further display characteristic age-dependent lipofuscin granules and modified cytoplasmic granularity (Moreno-Garcia et al., 2018; Singh Kushwaha et al., 2018). Lipofuscin accumulates in the cytoplasm, most likely in the lysosomes, and is detectable as autofluorescent granules, which consist of oxidized macromolecules and metal ions. Since microglia cells that contain lipofuscin are often seen in close contact to neurons, we might assume that neurons secrete or actively transport lipofuscin toward microglia, which will then take it up (Brunk and Terman, 2002). As in adult mice, microglia in aged mice show regional heterogeneity in the dynamics of their age-related changes in gene expression (Grabert et al., 2016; Masuda et al., 2019). Older animals exhibit increased mRNA levels of major histocompatibility complex (MHC) class II, cluster of differentiation 86 (CD86), class II, major histocompatibility complex trans-activator (CIITA), and interferon gamma (IFN-γ). At the same time, molecules that limit macrophage activation, such as interleukin-10 (IL-10) and cluster of differentiation 200 (CD200) are reduced (Sierra et al., 2007). Studies on mouse and human have highlighted that microglia in the aged brain ultimately show alterations in genes related to immune functions (e.g., interferon signaling and immunoreceptor expression), cell-adhesion and phagocytosis (Olah et al., 2018; Hammond et al., 2019). It was shown that the blockade of CD22, a sialic acid binding receptor which is upregulated in microglia during aging, not only restored phagocytosis, but it also improved the cognitive function in aged mice (Pluvinage et al., 2019). Since microglia acquire a more pro-inflammatory state with age, they are "primed" and respond much stronger to any kind of second stimulus. As a result, pro-inflammatory cytokines are released more rapidly and to a greater extent (Henry et al., 2009; Salvi et al., 2017). Microglial depletion and repopulation partially reverse the age-related transcriptomic profile. However, in response to a secondary LPS stimulation, microglia from aged mice still show an exaggerated response compared to adult mice, which hints on the role of the microglial microenvironment and the permanence of the pro-inflammatory phenotype in the aged brain (O'Neil et al., 2018). These data are in accordance with the idea that the aging brain displays a continuing, low-level, pro-inflammatory state,

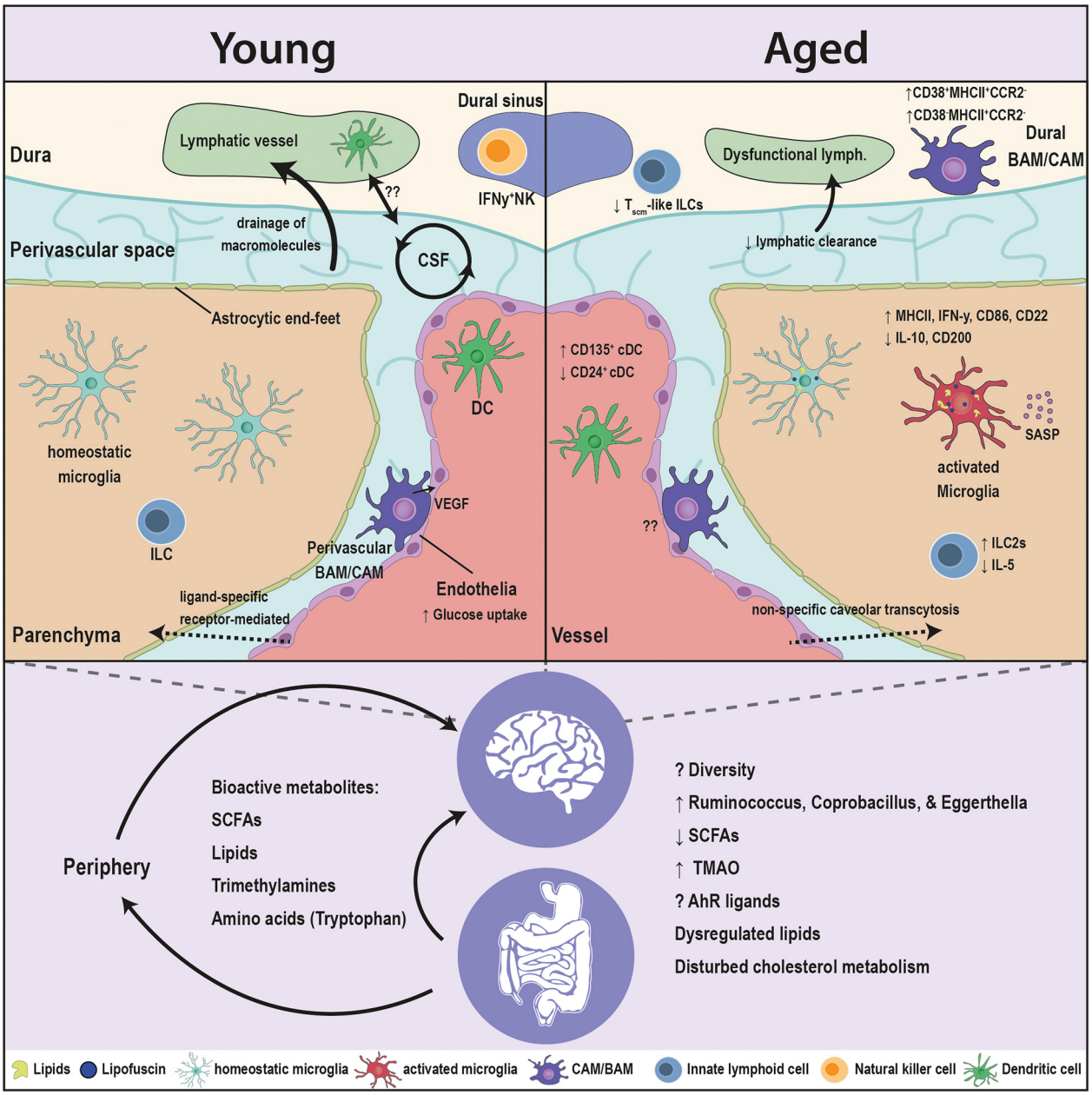

FIGURE 1 | Age-associated alterations in the brain innate immunity and the gut microbiota. Major crippling and life-threatening diseases, such as cancer, cardiovascular disease, and neurodegeneration, are all linked to advanced age. The innate immune cells are the first responders to insults in the brain, and are in constant communication with the microbiota. Aging has several facets on the brain, as well as the microbiota. The blood brain barrier (BBB) switches from ligand-receptor transport to a less specific caveolar transcytosis. The drainage of the CSF macromolecules declines and is associated to dysfunctional changes in the lymphatics and innate immune cells. Microglia, the brain resident macrophages, switch to an age-related phenotype in which microglia accumulate lipofuscin and lipid droplets, elicit a pro-inflammatory profile [elevation in major histocompatibility complex (MHC) class II, interferon gamma (IFN-γ), cluster of differentiation 86 (CD86), and cluster of differentiation 22 (CD22), and a reduction in interleukin-10 (IL-10) and cluster of differentiation 200 (CD200)], decreased phagocytic capacity and an induction of senescence-associated secretory phenotype (SASP). CNS- or border-associated macrophages (BAMs/CAMs) undergo numerous brain-maintaining functions in the healthy young brain that decline with age. BAMs/CAMs shit to $CD38^{+}MHCII^{+}CCR2^{-}$ and $CD38^{-}MHCII^{+}CCR2^{-}$ subsets in the aged brain. Classical dendritic cells (DCs) associated with the brain elicit an elevation in $CD135^{+}$ and a drop in $CD24^{+}$ subsets. Innate lymphoid cells (ILCs) switch from IL1C to IL2C transcriptomic profile and lessen the expression of the neuroprotective interleukin 5 (IL-5). Moreover, the microbiota interacts with cell populations of the brain both directly and indirectly through modulating the periphery. Multitudes of microbiota-derived molecules [short chain fatty acids (SCFAs), lipids, trimethylamines, amino acids] influence the activity of the brain innate immune cells and in turn modulate the brain functions. The levels of these bioactive metabolites are sensitive to age. The fine tuning of these molecules is disturbed in aging, due to compositional changes in the microbiota and the drop in the taxonomic diversity. Alterations in the microbiota are decisive to the dysregulation of immune function of the brain's innate immunity in aging and requires more comprehensive research.

which is termed "brain inflammaging." In fact, inflammaging results from chronic, low-threshold activation of the innate immune system, which can lead to a damaging, pathological situation (Franceschi et al., 2000). Innate immune cell functions decline with aging (Shaw et al., 2013). As a consequence of this immunosenescence one could imagine that cell debris of dying cells will not get removed in the aging brain by brain macrophages and slowly builds up to create a cell damaging environment. This enrichment of senescent cells is indirectly the incentive for the development of a typical proinflammatory phenotype by increasing levels of interleukins (IL) like IL-1, IL-6, and IL-18 or tumor necrosis factor-α (TNF-α), which are only present in low-level. The continuous presence of cytokines appears sufficient to inflict neuronal damage and ultimately forms the basis for age-related neurodegeneration (Lalancette-Hebert et al., 2007). In support of this assumption, the overall health status and even the life span could be dramatically improved when senescent cells were eliminated from aged mice (Baker et al., 2011; Zhu et al., 2015). In line with the later, a steady increase in lipid-laden cells (LLCs) is observed in the aging CNS. Perilipin (PLIN), a lipid droplet-associated protein, is a marker for autophagolysosome formation and indicates an increased lipophagic activity. Distinctive from lipofuscin, non autofluorescent Oil Red^+ $PLIN^+$ microglia show a lipid-laden multilocular pattern. The lipid-laden microglia expressed senescence markers and showed elevated TNF-α production and disturbed autophagy (Shimabukuro et al., 2016). In addition, isolated microglia, which showed lipid droplet accumulation, had higher oxidative stress and NAD^+/NADH ratio, implying metabolic defects. These lipid-accumulating microglia also elicited diminished phagocytosis, which was dependent on *GRN*, a gene that is implicated in frontotemporal dementia (Marschallinger et al., 2020). As microglia are environmental sensors that actively survey the CNS, age-related functional changes in microglia can be expected to also impact other CNS cells. For the senescent human brain it is not clear whether microglia are autonomously deteriorating or whether the observed morphological changes are merely a response to their changing microenvironment. But the age-related morphological changes most certainly affect other cells, especially neurons, which are directly contacted at their soma and synapses by microglia in response to the neuronal activation status (Lampron et al., 2013). They maintain brain homeostasis by responding rapidly to clear invading pathogens and tissue debris (Di Benedetto et al., 2017). It seems that the expression of several neuronal ligands, which can be sensed by microglial CX3C chemokine receptor 1 (CX3CR1) and CD200R receptors, decreases during the aging process and contribute to the sparking of a pro-inflammatory phenotype (Wu et al., 2015; Kabba et al., 2018). Moreover, microglia activation can lead to the secretion of soluble components that counteract apoptosis of oligodendrocytes, which is induced by TNF-α (Nicholas et al., 2002). Despite the age-related decline in surveillance function and the prominent activation of microglia, they still have the potential to upregulate genes promoting neuroprotection, secrete anti-inflammatory cytokines and regulate adaptive processes such as tissue repair (Bellver-Landete et al., 2019). The parallel modulation of both pro- and anti-inflammatory factors suggests that microglial cells are still able to self-regulate and restore homeostasis.

CNS- or Border-Associated Macrophages

At CNS interfaces with the periphery, i.e., meninges, perivascular space, and choroid plexus, another population of macrophages exists, collectively referred to as border- or CNS-associated macrophages (BAMs or CAMs). BAMs/CAMs are not only distinguishable from microglia by morphology and location, but they also do not express the typical microglia markers, e.g., P2RY12 and TMEM119, yet, they show high levels of the class B scavenger receptor CD36 and the mannose receptor CD206. Similar to microglia, studies have shown that BAMs/CAMs subsets share a yolk sac origin, are long lived and exhibit only minor replenishment by circulating peripheral progenitors, except for dural macrophages and stromal macrophages. This choroid plexus subset of macrophages is postnatally substituted by bone marrow-derived progenitor cells (Kierdorf et al., 2019). Despite the distinctive location within specialized anatomical structures, thorough investigations have only recently started to unravel the heterogenous subset identities and physiological functions of BAMs/CAMs (Van Hove et al., 2019). For example, in the brain meninges, the pia matter predominantly harbored $Lyve1^+MHCII^+$ BAMs/CAMs, while subsets residing in the dura matter lacked Lyve1 expression (Mrdjen et al., 2018). At the interface between the bloodstream and the parenchyma, perivascular and meningeal macrophages monitor and filter the cerebrospinal fluid (CSF), and scavenge antigens and possibly harmful substances (Serrats et al., 2010; Nayak et al., 2012). Indeed, recent studies have underscored the potential role of macrophages along the dural sinuses in antigen presentation and immune surveillance of the lymphatic drainage. Here, it is important to note that in old animals the lymphatic drainage of CNS antigens was decreased in relation to younger animals (Louveau et al., 2018; Rustenhoven et al., 2021), yet the role of BAMs/CAMs in this process is unclear. BAMs/CAMs display specific alterations in the aged brain, which are associated with a heightened proinflammatory cytokine profile and antigen presentation to T cells. The number of $CD38^+MHCII^+CCR2^-$ and $CD38^-MHCII^+CCR2^-$ subsets increased in aged mice, while $CD38^+MHCII^-CCR2^-$ and $CD38^-MHCII^+CCR2^+$ subsets decreased (Mrdjen et al., 2018). Studies have proposed that the interaction between perivascular macrophages and other cellular components of the blood brain barrier (BBB) could regulate the BBB integrity and vascular permeability. Perivascular macrophages secrete vascular endothelial growth factor (VEGF) to regulate glucose uptake by brain endothelial cells (BECs) (Jais et al., 2016). BECs, key players of the BBB, are sensitive to age. BECs, specifically the capillaries, undergo age-related transcriptional changes, which are dependent on the age identity of the circulatory proteins and have known associations with brain aging and AD (Chen et al., 2020).

Neutrophils and Dendritic Cells

Under homeostatic conditions, additional innate immune cells present at the interface of the CNS include neutrophils, and two DC subsets: classical (cDCc) and plasmacytoid (pDCs). Despite being restricted by the BBB from the brain parenchyma and CSF, neutrophils and DCs were shown to be modulating the CNS in the course of different pathologies like infections, trauma, autoimmune disorders or neurodegeneration. Neutrophil infiltrates secrete free oxygen radicals (ROS) and proteolytic enzymes (i.e., MMP-9) that damage the BBB and potentially injure neurons (Manda-Handzlik and Demkow, 2019). DCs are the major antigen presenting cells in the CNS due to the expression of MHCII. Upon inflammation, resident DCs are augmented with monocyte-derived DC (moDC), which differentiate from infiltrating Ly6C^{hi} monocytes and can be detrimental for the CNS. Depletion or inactivation of moDCs ameliorates clinical disability in mouse models of Multiple sclerosis (Giles et al., 2018). In aged mice, the frequency of neutrophils had high variations, but pDCs showed a robust drop. Moreover, the number of cDC subsets CD24^{+} cDC2s declined, while the amount of CD135^{+} cDC2s rose (Mrdjen et al., 2018). Detailed transcriptomic and functional studies on neutrophils and cDCs at the interfaces of the aging CNS are still lacking; however, based on their role in phagocytosis and antigen presentation, it is tempting to speculate on their involvement in the lymphatic drainage from the CNS.

Innate Lymphoid Cells

Innate lymphoid cells (ILCs) are a rapidly expanding group of cells that are critical to the innate immune system. ILCs are tissue resident cells that show high organ-specific plasticity. These cells also stem from lymphoid progenitor as B and T lymphocytes and respond as one of the first cells to stimuli in their close vicinity but lack the ability to recognize antigens (Spits et al., 2013). ILCs are currently subdivided into three subclasses, which inherently mimic subsets of T-helper (Th) cells in their cytokine production and functions. The response of type 1 ILCs (ILC1s) is similar to Th1 cells. Parallel to Th2 cells, ILC2s produce cytokines like IL-4, IL-5, and IL-13, which typically contribute to allergic inflammations. When activated, ILC3s mimic Th17 cells by secreting IL-17 and IL-22 (Vivier et al., 2018). NK cells can be seen as cytotoxic ILCs and are very similar to ILC1s (both are CD3^{-}NK1.1^{+}) when it comes to phenotype and function. The main difference between ILC1s and NK cells can be found in proteins that contribute to cell retention into the tissues (i.e., CD49a, CD103, and the lectin CD69) (Romero-Suárez et al., 2019). Functionally, the peripheral role of ILCs has been profoundly investigated in the last decade. In particular, their immune response against allergens, viruses, and inflammation of the lung and intestine was carefully characterized (Vivier et al., 2018). ILC1s are mainly found in the choroid plexus of the brain, in meninges and within the brain parenchyma where only few NK cells are localized. ILC2s are long-lived but less abundant and are present in the meninges and the choroid plexus but are absent in the brain parenchyma (Gadani et al., 2017). In the aged brain, ILCs elicited a shift toward an ILC2 transcriptional profile and showed an increased abundance of ILC-like cells, which express a T memory stemness (T_{scm}) signature (Golomb et al., 2020). Interestingly, through the reduction of TNFα and the enhancement of hippocampal neurogenesis, the intracerebroventricular transfer of activated ILC2 or the direct administration of IL-5, a major ILC2 product, clearly improved the learning and memory abilities in old mice (Fung et al., 2020). The recent findings on ILCs highlight one avenue where the innate immune cells in the CNS counteract the age-associated functional decline.

Immune surveillance systems work together, to keep the brain in a state of homeostasis. The expansion of neurological injury, which leads to neurodegeneration, may be favored by the endogenously advancing regression in immune effector activity. Furthermore, the CNS-resident and -associated innate immune cells' constant immune surveillance makes them an important communication-axis between the microbiota and the CNS.

THE AGING GUT MICROBIOME

Both the host and the microbiota undergo age-related changes, and host–microbiota interactions may represent a pivotal component in aging. In several studies the analysis of numerous stool samples indicated that the overall composition but also the diversity of the microbiota found in the gut changes with age (Claesson et al., 2011; Lakshminarayanan et al., 2014). Indeed, a healthy microbiota could support human longevity (Badal et al., 2020), and a young-microbiota transplant could prolong the lifespan in fish (Smith et al., 2017). Although the current dogma holds that the gut microbiota of aged individuals becomes less diverse and variable with advancing age (Claesson et al., 2011). It is important to point out that changes in the microbiota of the elderly may derive from dietary changes, prolonged intestinal transit times, lack of physical activity, residency in elderly homes, hospitalization, recurrent infections and frequent use of antibiotics and other medications. One study showed that these changes in diversity do not depend on the chronological age but are rather influenced by biological or functional age (Maffei et al., 2017). On the other hand, a study on a Japanese population, which included subjects ranging from new-born babies to centenarians, has shown an age-dependent increase in the microbiota diversity that only dropped in the centenarian group (Xu et al., 2019). This leaves the link between microbiota diversity and aging obscure. Vicissitudes in the microbiota during aging are certainly causally connected to inflammaging, as a lasting activation of the immune system contributes to immunosenescence. A prolonged state of inflammation could make the host more susceptible to potentially disadvantageous bacteria, which in turn contribute to the progression of various pathological conditions in older adults (Bischoff, 2016). It should be mentioned that in this context the human gut ecosystem is the best-studied microbiome. The reason is its ubiquitous role in converting environmental signals like nutrients from food into bioactive compounds that send signals to other organs including the brain. These bioactive compounds enable gut bacteria to interact and potentially modulate the function of the hosts'

central nervous system. One can imagine that this kind of interface is not only formed by the microbiota of the gut. There are also reports on the aging microbiome in the lung, vagina, and urogenital tract and the possibility that bacteria from these niches have an indirect impact on brain function (Janiak et al., 2021; Wilmanski et al., 2021). To interpret these results, it seems that with increasing biological age, the diversity of gut microbiota shifts and at the same time specific microbial taxa emerge that are linked to unhealthy aging.

HOW GUT MICROBIOTA INTERFERES WITH INNATE IMMUNE CELLS OF THE AGED BRAIN

The interaction between the gut microbiota and the innate and adaptive immune systems through direct engagements at mucosal surfaces or microbiota derived metabolites is unambiguous. The peripheral immune system is quite sensitive to slight alterations in the circulating metabolites and plasma cytokine composition, which can result due to microbiota dysbiosis (Zheng et al., 2020). Intriguingly, parabiosis or plasma transfer experiments that expose a young animal to old blood decreases hippocampal neurogenesis, promotes microgliosis and, ultimately, impairs learning and memory function (Villeda et al., 2011). On the other hand, exposing aged animals to young blood improves the cerebral vasculature, enhances neurogenesis in the subventricular zone and ameliorates the decline in olfaction (Katsimpardi et al., 2014). The brain has long been thought to be immune-privileged. However, the test of time has proved this terminology not absolute. Under homeostatic conditions, the degree of immune-privilege varies depending on age and neurological health. Additionally to the aforementioned age-associated alteration of the microbiota in aging, the neurovascular unit of the BBB undergoes a transition in caveolar transcytosis from ligand-specific receptor-mediated to a non-specific mode, which could potentially allow atypical primary or secondary microbiota-derived molecules uptake into the CNS (Yang et al., 2020). Indeed, beyond peripheral immunity, microbiota-derived signaling molecules have been implicated in CNS immunity, neuropsychiatric and neurodegenerative disorders (Mossad and Erny, 2020).

Compared to other understudied CNS innate immune cells, the microbiota-microglia axis has been well investigated during development and adulthood (Mezö et al., 2019). In a time-dependent and sexually dimorphic manner, germ-free (GF) mice, acute antibiotic intervention, or a lack of taxonomic sophistication hinder the development and immune response of microglia (Erny et al., 2015; Thion et al., 2018). Recolonization with complex microbiota or dietary supplementation with short-chain fatty acids (SCFAs) seems to reverse these defects, as activation of the SCFA receptor FFAR2 restores microglial maturation (Erny et al., 2015). These findings indicate that maintaining microglia homeostasis requires a low level of subclinical immune activation through the microbiota. In line with previous reports, a recent study on aged mice has shown that microglia and BAMs/CAMs in aged mice shift toward a pro-inflammatory and antigen presentation phenotype, however, when antibiotics were applied for only a short time period, gut-dysbiosis had little impact on microglia and BAMs/CAMs homeostasis (Golomb et al., 2020). Another interesting finding on the interaction of the microbiota with macrophages during aging comes from a study on peripheral macrophages. Fecal microbiota transplantation (FMT) from aged mice to young GF recipients elevates circulating inflammatory cytokines and impairs macrophage phagocytosis (Thevaranjan et al., 2017). Furthermore, for ILCs, antibiotics treatment specifically reduced T_{scm}-like ILCs transcriptome signature only in the aged mice (Golomb et al., 2020). Nevertheless, the functional relevance of the T_{scm}-like ILCs in the aging brain remains unclear. Furthermore, the gut microbiota regulates IFNγ expression in meningeal NK cells. Under neuroinflammatory conditions, the IFNγ^+ NK cells maintain a subset of astrocytes (LAMP1$^+$TRAIL$^+$), which reduces inflammatory processes in the CNS by initiating T cell apoptosis (Sanmarco et al., 2021).

Age-Associated Gut Borne Metabolites

Since the diet, drug intake, lifestyle, and gut-microbiome composition all change as people age, the intestinal metabolic environment, or the levels of microbial metabolites, will eventually change as well.

SCFAs

Short-chain fatty acids (SCFAs) belong to the group of saturated fatty acids and result from fermented dietary fibers in the gut. Butyrate, propionate and acetate constitute the majority of SCFAs and have significant impacts on several physiological features and help to maintain the integrity of the gut barrier, they contribute to metabolism in the periphery and support gut homeostasis (Donohoe et al., 2011; Den Besten et al., 2013; Thorburn et al., 2014). Surprisingly, microbial signatures expressing high concentrations of SCFA producers have been found in many studies investigating the microbiota of normal and aged animals and humans (Kong et al., 2016), yet the fecal concentration of SCFAs decreases with age (Woodmansey et al., 2004). Other factors that can influence SCFA fecal levels include the carbon present in diet or the absorption/excretion of these metabolites, which may be impaired in older subjects. Low levels of bacterially derived SCFAs in the aged microbiota are partially to blame for the enhanced immune status and worse outcomes in aged mice after a stroke. SCFAs were restored to the level of the young microbiome by SCFA-producing bacteria (probiotics) and a food supply (prebiotics) for these bacteria, and stroke outcomes improved dramatically. Of importance, these effects on outcome were not related to the chronological age of the mice but to the age of the microbiome (Lee et al., 2020). As SCFAs were found to mimic features of the microbiota's influence on microglia under homeostasis, the role of SCFAs on microglia in CNS disorders has recently gained increasing attention. For instance, GF-housed APP/PS1 transgenic mice that receive SCFAs show increased cerebral Aβ loads and a specific microglial activation that is caused by enhanced ApoE-Trem2 signaling (Colombo et al., 2020).

Lipids

As previously stated, dysregulation of lipid metabolism and intracellular accumulation of certain lipid moieties are hallmarks of microglia in the aged brain (Marschallinger et al., 2020). The microbiota has an effect on the lipid composition of metabolically vital organs including adipose tissue, liver, and lipid-rich organs like the retina, according to evidence from GF animals (Backhed et al., 2004; Orešič et al., 2009; Velagapudi et al., 2010). The brain displays high lipid content and a variety of different lipid categories (Han, 2007). With increasing age, the lipid composition of the brain is changing (Yu et al., 2020). Changes in cholesterol metabolism, for example, have been linked to age-related neurodegenerative disorders and cognitive impairment (Zhang and Liu, 2015). GF animals populated with an aged microbiota show changes in lipid profiles in the cortex and liver, which is intriguing (Albouery et al., 2020). Whether the aged-microbiota mediated dysregulation of lipids in the CNS extrapolates to the lipid-laden microglial phenotype in the aged brain or further affects other innate immune cells remains unexplored.

Trimethylamines

Dietary cholines are metabolized in the gut to form trimethylamines. In a first step, choline is broken down into trimethylamine (TMA), which is transported to the liver where it is converted into trimethylamine-N-oxide (TMAO) (Bennett et al., 2013). Increased plasma levels of TMAO affect cholesterol metabolism and are linked to a higher risk of several age-related diseases including cardiovascular disease, colorectal cancer and atherosclerosis (Koeth et al., 2013; Bae et al., 2014). Since TMAO is found in the brain it is most likely able to cross the BBB (Del Rio et al., 2017). While controlling for age, sex and APOE ε4 genotype, higher CSF levels of TMAO were detected in AD and mild cognitive impairment patients compared to healthy controls suggesting the potential involvement of TMAO in AD (Vogt et al., 2018). Moreover, plasma TMAO levels, in an aging cohort, were inversely correlated with cognitive functions through promoting pro-inflammatory signaling in astrocytes and microglia (Brunt et al., 2021).

Amino Acid Metabolites

Human metagenomic studies suggest the gut microbes to be largely involved in amino acid metabolism. Aromatic amino acids, i.e., tryptophan, are majorly supplied in humans through diet or produced by the microbiota. By stimulating astrocytes, bacterial conversion of tryptophan to indoles, ligands for the aryl hydrocarbon receptor (AhR), can reduce brain inflammation and restrict disease severity in MS mouse models (Rothhammer et al., 2016). AhR signaling in microglia fine-tunes the microglial expression of TGFα and VEGF-B, which in turn should regulate astrocyte activation (Rothhammer et al., 2018).

CONCLUSION AND FUTURE PERSPECTIVES

There is an evident gap in understanding the direct and indirect links between the microbiota and CNS innate immune cells other than microglia. This gap is even wider when it comes to investigating these interactions in the context of aging. Studies utilizing GF mice, acute antibiotic treatments, and fecal microbiota transplantations from the different ages, can be combined with different genetic models or innate immune cell pharmacological targeting approaches in order to gain some insights into the age-associated impact of the microbiota. Combinations with deep NGS approaches are essential to understand the specific phenotype alterations that occur in the CNS innate immune cell populations upon microbiota dysbiosis. Moreover, extensive unbiased analyses of the microbiota-derived metabolites and peptides at different sites enroute to the CNS are required to understand the dynamics of these molecules and their specific effects on CNS functions. More importantly, major studies on aging human populations expanding on the integrative human microbiome project (Proctor et al., 2019), which dwells into the associations between the microbiota's identity and the changes in circulating molecules in blood and CSF, can provide the translatable link between the microbiota-derived signaling molecules and the innate immune cells of the CNS.

In conclusion, it is difficult to comprehend the biological and molecular basis of senescence, as well as the interplay between microglial senescence and the gut microbiota regulating various functions in the healthy and diseased brain. This, however, represents a therapeutic opportunity that could lead to the discovery of new pharmacological targets for maintaining or restoring physiological tasks in long-lived individuals.

AUTHOR CONTRIBUTIONS

Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Effect of a Multispecies Probiotic Mixture on the Growth and Incidence of Diarrhea, Immune Function, and Fecal Microbiota of Pre-weaning Dairy Calves

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The effects of different doses of a multispecies probiotic (MSP) mixture on growth performance, the incidence of diarrhea rate and immune function, and fecal microbial diversity and structure were evaluated in pre-weaning Holstein dairy calves at WK2, WK4, WK6, and WK8. Forty Chinese Holstein female newborn calves were randomly assigned to four treatments with 10 calves in each group, C (control group), T1 (0.5 g MSP/calf/day, T2 (1 g MSP/calf/day), and T3 (2 g MSP/calf/day) groups. The experimental period was 56 days. Feed intake and health scoring were recorded every day until the end of the experiment. Fecal contents and blood samples were sampled at WK2, WK4, WK6, and WK8. Growth performance, incidence of diarrhea, and total serum concentrations (IgA, IgG, and IgM) were analyzed. Bacterial 16S rRNA and fungal ITS genes were high-throughput sequenced for fecal microbiota. The relationships among the populations of the principal fecal microbiota at WK2 and the growth performance or serum immunoglobulin concentrations were analyzed using Pearson's rank correlation coefficients. The MSP supplementation reduced the incidence of diarrhea in the first 4 weeks of life, and serum IgA, IgG, and IgM concentrations increased between WK2 and WK8 in the T3 group. There was an increase in growth performance and reduction in the incidence of diarrhea until WK4 after birth in T3 group, compared with the control, T1, and T2 groups. The results of fecal microbiota analysis showed that *Firmicutes* and *Bacteroides* were the predominant phyla, with *Blautia*, *Ruminococcaceae_UCG-005*, *norank_f__Muribaculaceae*, *Bacteroides*, *Subdoligranulum*, and *Bifidobacterium* being the dominant genera in calf feces. *Aspergillus*, *Thermomyces*, and *Saccharomyces* were the predominant fungal phyla. Compared with the control, in T1 and T2 groups, the MSP supplementation reduced the relative abundance of *Bacteroidetes* and increased the relative abundance of *Bifidobacterium*, *Lactobacillus*, *Collinsella*, and *Saccharomyces* at WK2 in group T3. Thus, the fecal microbial composition and diversity was significantly affected by the MSP mixture during the first 2 weeks of the calves' life. MSP mixtures reduced the incidence

of diarrhea in pre-weaning calves (during the first 4 weeks of life). There was a significant improvement in growth performance, reduction in calf diarrhea, balance in the fecal microbiota, and an overall improvement in serum immunity, compared with the control group. We, therefore, recommend adding 2 g/day of multispecies probiotic mixture supplementation in diets of dairy calves during their first 4 weeks of life before weaning.

Keywords: multispecies probiotic, growth, diarrhea, microbiota, calves

INTRODUCTION

Neonatal diarrhea occurs frequently in dairy calves all over the world, causing huge economic and productivity losses that undermine healthy and sustainable development of animal husbandry (Donovan et al., 1998, 2002; El-Seedy et al., 2016). Moreover, even if calves recover from the diarrhea, their subsequent growth and development are hindered, which later affects their productivity in adulthood (Heinrichs and Heinrichs, 2011). Generally, feed supplementation could reduce the incidence of diarrhea and improve the health of calves. Therefore, it is very important to determine the application of effective antidiarrheal agents (Caruso, 2018; Wang et al., 2018; Zhao et al., 2018) in dairy farming since the European Union (Casewell et al., 2003) and China (Ministry of agriculture and rural areas, 2019) prohibited the use of antimicrobial growth promoters.

In 2014, the International Association of Probiotics and Prebiotic Sciences (ISAPP) emphasized the importance of probiotics in improving the survivability of animals (Markowiak and Śliżewska, 2018). Probiotics are defined as "living organisms that bring health benefits to the host at an appropriate dose" (Hill et al., 2014). Multispecies probiotics (MSPs) were more effective than single-strain probiotics, especially in treating antibiotic-associated diarrhea in children (Ki et al., 2012; Łukasik and Szajewska, 2018), improving animal growth performance (Renaud et al., 2019), resisting bacterial infection (Perdigon et al., 1990; Avila et al., 1995; Lema et al., 2001; Woof and Kerr, 2006; Ehrenstein and Notley, 2010; Collumbien et al., 2012; Crassini et al., 2018), weight gain after stimulation post-enteritis (Renaud et al., 2019), and improving intestinal microbiota (Cruywagen et al., 1996; Chen et al., 2015; He et al., 2017; Hod et al., 2018; Biagioli et al., 2019).

Multispecies probiotics [*Lactobacillus acidophilus* (McFarland et al., 2018; Łukasik and Szajewska, 2018), *Bacillus subtilis* (Rui and Ma, 2020), *Saccharomyces cerevisiae* (Thévenot et al., 2015)] have achieved certain results in human application, and there are similar reports in animals. Studies have found that *Lactobacillus acidophilus* (Sharma et al., 2018), *Bacillus subtilis* (Sun et al., 2010; Zhang et al., 2017; Wood et al., 2019), and *Saccharomyces cerevisiae* (Fomenky et al., 2018; Villot et al., 2019) can improve calf growth performance by improving immune function and balancing the structure of intestinal microbiota. The objectives of this study are: (1) To evaluate whether MSP supplementation can reduce the incidence of diarrhea in pre-weaning calves while improving the growth performance. (2) To evaluate whether the MSP supplementation can improve serum immunity (IgA, IgG, and IgM) in pre-weaning calves. (3) To evaluate whether MSP supplementation can affect the diversity and composition of the fecal microbiota of pre-weaning calves.

MATERIALS AND METHODS

This study has been approved by the ethics committee of the College of Animal Science and Technology, Shihezi University (No. A2019-155-01).

Preparation of the Multispecies Probiotics Mixture

Probiotic strains of *Lactobacillus acidophilus S5* (Wu, 2013), *Bacillus subtilis* No. Bzg988118 (Bao, 2013), and *Saccharomyces cerevisiae SHZ2017* were provided by the Biological Feed Laboratory of the College of Animal Science and Technology, Shihezi University, China. *In vitro* analyses revealed that all three strains have the potential benefits of probiotics, inhibiting the growth of Gram-positive and Gram-negative pathogens (i.e., *Escherichia coli* K99, *Salmonella*, and *Staphylococcus aureus*), resist low pH and bovine bile salts, and tolerance to artificial gastrointestinal environment (Wu et al., 2021).

Each of the three strains were cultured, respectively, in de Man, Rogosa, and Sharpe medium (MRS), yeast peptone dextrose (YPD), and Luria–Bertani (LB) medium (purchased from Qingdao Gaokeyuan Haibo Biotechnology Co., Ltd., Qingdao, China), where *L. acidophilus* was anaerobically cultured at 37°C for 20 h, while *S. cerevisiae* and *B. subtilis* were cultured on a shaker at 37°C for 20 h, as described by Dong et al. (2013) after cultivation. One liter of the bacterial culture enrichment was centrifuged at 4,000 rpm for 3 min to remove the bacterial supernatant. The precipitation was washed with 60 ml of sterile PBS buffer including 5% glycerol and 20% skim milk powder (Shu et al., 2015), then mixed with 0.25 kg bran and freeze dried. The ratio of the three strain probiotics complex was 3:3:1, representing *L. acidophilus*, *B. subtilis*, and *S. cerevisiae* fermentum based on previous research (Wu et al., 2021).

Animals and Diet

Forty Chinese Holstein female calves (age = 6 ± 3 days, BW = 40.86 ± 2.65 kg) were selected and randomly assigned into four treatment groups with 10 calves per group. All the

calves were removed from their dams immediately after birth and housed in individual pens (1.8 × 1.4 × 1.2 m), which were bedded with straw and had iron fences to avoid cross-contamination for the entire length of the experiment (December 2019–February 2020). Calves were fed 4 L of colostrum (pasteurized at 60°C for 1 h) from a bottle within 1 h of birth. The calves were fed twice with milk in two equal-volume plastic buckets daily, at 0700 and 1800 h. On day 5, the volume of feed was increased to 6 L/day (3 L/meal) of milk, which was produced in the same farm and pasteurized at 60°C for 1 h. On day 6, the volume of feed was increased again to 7 L/day (3.5 L/meal), and finally, on day 7 to 53, the volume of feed was 8 L/day (4 L/meal), which was gradually reduced to zero by 1 L/day until weaning at day 61. Starter concentrates were provided by Xinjiang Urumqi Zhengda Feed Co., Ltd. (Urumqi, China) and was fed to the calves from day 4. All calves received the same colostrum and milk. The MSP (1 g MSP contains *L. acidophilus* 3×10^9 CFU, *B. subtilis* 3×10^9 CFU, and *S. cerevisiae* 1×10^9 CFU) was prepared by the Biological Feed Laboratory of the College of Animal Science and Technology, Shihezi University (Wu et al., 2021). The ingredients and chemical composition of the starter concentrates are shown in **Table 1**.

Experimental Design and Sample Collection

The MSP was provided in the form of freeze-dried powder and was mixed with fresh cow's milk. The control calves were fed with starter and milk that was not supplemented with MSP, while the calves in the treatment (T) groups received MSP: T1 at 0.5 g/calf/day, T2 at 1 g/calf/day, and T3 at 2 g/calf/day. No additives were fed to the control group. Before the start of the 8-week trial experiment, animals were individually checked for signs of disease, injury, and dehydration, and those that were initially deemed unhealthy were not included as part of the 40 calves used. The trial lasted for 56 days, during which all the animals had free access to the same fresh water and starter concentrate. This study was conducted between December 2019 and February 2020 at Shurui Farm, Tianshan Co., Shihezi, China.

TABLE 1 | Ingredient composition and nutrient levels of starter (DM basis).

Item	Value
Ingredient, g/kg of DM	
Corn	55.20
Soybean meal[1]	18.50
Corn gluten meal	10.00
DGGS[2]	13.00
Limestone	1.80
NaCl	0.50
Premix[3]	1.00
Total	100.00
Chemical analysis	
DM, g/kg	87.33
CP, g/kg	19.92
Ether extract, g/kg	4.64
ADF, g/kg	6.02
NDF, g/kg	16.53
Ash, g/kg	5.38
Calcium	1.15
Phosphorus	0.58

[1] *Soybean meal: 89.1% DM and 42.6% CP.*
[2] *DDGS, distiller's dried grains with solubles are the nutrient-rich co-product of dry-milled ethanol production.*
[3] *Premix provides the following per kg of the starter diet: VA 15,000 IU, VD 5,000 IU, VE 50 mg, Fe 90 mg, Cu 12.5 mg, Mn 30 mg, Zn 90 mg, Se 0.3 mg, I 1.0 mg, and Co 0.5 mg.*

Blood samples were obtained from six calves per group by jugular vein puncture using 10-ml of gel vacuum tubes on the morning of WK2, WK4, WK6, and WK8. Samples were centrifuged at 3,000 × *g* for 15 min at 4°C using a high-speed refrigerated centrifuge Eppendorf 5810R (Eppendorf AG, Hamburg, Germany). Separated serum was stored at −20°C for subsequent total serum IgA, IgG, and IgM measurements.

Fecal and blood samples were collected from the same six calves per group at WK2, WK4, WK6, and WK8 using sterile gloves after feeding for 3 h. Before collecting the samples, centrifuge tubes (Corning, NY, United States) were sterilized in an autoclave and then used to collect the fresh feces. A rectal palpation method was used to collect a stool sample (20 g) directly from the rectum. During sampling, the sample was stored in a 15-ml cryovial on ice with about 0.2 g of feces being collected into each 2-ml tube. A total of four tubes were filled for six animals from each group. These fecal samples were snap-frozen in liquid nitrogen and stored at −80°C for later analyses.

Analysis of Growth Performance and the Incidence of Diarrhea

The average daily gain (ADG) was calculated by weighing the calves on days 1 and 56. The DMI of milk and starter was also recorded throughout the trial. The starter was sampled for analyses of DM content (AOAC International, 2005; method 930.15), CP (AOAC International, 2000; method 976.05), and ether extract (AOAC International, 2003; method 4.5.05) using standard procedures of the AOAC International. The NDF and ADF contents were determined as described by Van Soest et al. (1991).

A standard health scoring system (Renaud et al., 2018) was used for the fecal scores every morning at 1000 h. In short, fecal consistency was scored on the following scale: 0 = normal, 1 = half-shaped and pasty, 2 = loose but staying on the mat, and 3 = watery, sieve through the mat. A case of diarrhea is defined when the fecal score is at least 2 (Lesmeister et al., 2004). The following formula was used to calculate the incidence of diarrhea in each group.

Incidence of diarrhea% = calves with diarrhea in each group × diarrhea days/(total calves in each group × experimental days) × 100%.

Analysis of Total Serum IgA, IgG, and IgM Concentrations by ELISA

The total serum IgA, IgG, and IgM antibody concentrations were determined using bovine ELISA kits purchased from NanJing JianCheng Bioengineering Institute (Nanjing, China). All tests were run according to the manufacturer's protocols.

Bacterial 16S rRNA Gene and Fungal ITS Gene High-Throughput Sequencing

Genomic DNA from the microbial community in the fecal samples was extracted by using the E.Z.N.A.® soil DNA kit (Omega Bio-Tek, Norcross, GA, United States) according to the manufacturer's protocol. The extracted DNA was checked on a 1% agarose gel, and DNA concentration and purity were determined using a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, NC, United States).

The primer sequences for the amplification of bacterial V3–V4 region are: F: 5′-ACTCCTACGGGAGGCAGCAG-3′ and R: 5′-GGACTACHVGGGTWTCTAAT-3′ (Chang et al., 2020). PCR conditions were as follows: 95°C for 3 min; 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, followed by a hold at 72°C for 10 min. The primer sequences for the amplification of fungal ITS1F–ITS2R region amplification are F: 5′-CTTGGTCATTTAGAGGAAGTAA-3′ and R: 5′-GCTGCGTTCTTCATCGATGC-3′ (Hoffmann, 2013). PCR conditions were as follows: 95°C for 3 min; 27 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s; followed by a hold at 72°C for 10 min. The sequences were submitted to GenBank, and the accession number obtained is BioProject ID: PRJNA692054.

Illumina MiSeq Sequencing

Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq PE300 platform/NovaSeq PE250 platform (Illumina, San Diego, CA, United States) following the standard protocols by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

Processing of Sequence Data

The key steps of sequencing data analysis are briefly described here. To get clean reads, the raw data had to be preprocessed to eliminate adapter contamination and low-quality data. Sequence data was quality filtered using FastQC version 0.20.0 (Chen et al., 2018) and merged using FLASH version 1.2.7 (Magoč and Salzberg, 2011). The bacterial and fungal tags were clustered into operational taxonomic units (OTUs) by the QIIME (v7.1) software based on 97% sequence similarity using the UPARSE script (Edgar, 2013). Representative OTU sequences of fungi were classified using the ribosomal database project (RDP) classifier v.2.2 based on the UNITE database (Abarenkov et al., 2010). Then Majorbio cloud software was used to perform several key analyses, including α diversity (including observed species, Shannon, Simpson index, Ace, and Chao1), species composition (heatmap, bar), beta diversity (beta), diversity analysis (including PCA), linear discriminant analysis effect size (LEfSe), and association analysis.

Statistical Analysis

The Durbin–Watson test was used to check the randomness of the initial and final BW data to test that the randomization had been effective. A chi-squared contingency test was used to compare the effect of MSP on the prevalence of diarrhea. The growth performance data was analyzed using a one-way ANOVA in the MIXED procedure of SAS 9.4. The serum immunoglobulin concentration and fecal microbial data were analyzed on the basis of repeated measurements, and a compound symmetry variance and covariance structure using the GLIMMIX procedure of SAS 9.4. The repeated measures model contained fixed effects of treatment, day, and the interaction of treatment and day, and the random effect of calf identity. The data are presented as the least squares mean and standard error of the mean. Differences between the treatment groups were identified using Tukey's multiple range test. A $p \leq 0.05$ was accepted as statistically significant, and p-values between 0.05 and 0.10 were considered to represent a statistical trend. The relationships between the populations of the principal fecal microbes on day 14 and the growth performance or serum immunoglobulin concentrations were analyzed using Pearson's rank correlation coefficients.

RESULTS

Performance and Incidence of Diarrhea

No differences were observed in the initial or final BW of the calves in the four groups. Supplementation with MSP in group T2 significantly increased the ADG of calves when compared with the control group ($p < 0.05$; **Table 2**). No differences were observed in the DMI of starter or in the feed efficiency between the four groups. However, total feed intake by groups T1, T2, and T3 were much higher than that of the control group ($p < 0.05$). There was a slight reduction in the incidence of diarrhea in calves in group T3 when compared with the control group at WK2 (12.14 vs. 25.11; $p = 0.02$). In addition, supplementing with MSP reduced the incidence of diarrhea during the first 4 weeks of life in group T3 compared with the C, T1, and T2 groups ($p = 0.02$; $p = 0.04$).

Total Serum IgA, IgG, and IgM Concentrations

The total serum IgA and IgM concentrations in group T3 were significantly higher than those in the control group between WK2 to WK8 ($P < 0.05$; **Table 3**). The total serum IgG concentration in group T3 was also significantly higher than that in the control group ($p < 0.05$) between WK4 to WK6. However, no differences were observed in the concentrations of IgA and IgM in the control group and group T1 between WK2 to WK8 (**Table 3**); no differences were observed in the concentrations of IgG between groups T1, T2, and control group at WK2 (**Table 3**). In addition, treatment and time had a significant effect on serum IgA, IgM, and IgG concentrations, but no differences were identified with respect to the interaction of treatment and time.

Fecal Microbial Diversity

In this study, 16S rRNA and ITS genes were amplified, and their sequences were analyzed to study the effect of probiotics on fecal microbiota of pre-weaning calves during the four time points (WK 2, 4, 6, and 8). A total of 2,907,932 high-quality sequences were obtained from the rectal samples, with an average of 37,862 sequences per sample (26,220–43,090 sequences).

TABLE 2 | The growth performance and incidence of diarrhea in Holstein dairy calves fed with different doses of multispecies probiotics.

Items	Treatment (Trt)[1]				SEM	*p*-Value
	C	T1	T2	T3		Trt
Initial BW, kg	41.12	42.22	41.44	41.16	2.65	0.28
Final BW, kg	82.96	87.16	89.12	86.98	2.99	0.10
ADG, g/d	664.29^{b}	815.71^{ab}	875^{a}	720^{b}	27.69	0.03
Starter intake, g of DM/d	24.20^{b}	29.58^{a}	28.66^{a}	30.82^{a}	0.67	0.08
Total feed intake, g of DM/d	1,241.61^{b}	1,306.44^{a}	1,295.35^{a}	1,321.44^{a}	8.1	<0.01
Feed efficiency, g of DMl/g of gain	1.34	1.25	1.34	1.44	0.03	0.61
Incidence of diarrhea (d 1 to d 14), %	25.11	20	20	12.14	0.049	0.02
Incidence of diarrhea (d 15 to d 28), %	1.14	1.13	1.85	–	0.041	0.04
Incidence of diarrhea (d 29 to d 42), %	–	1.13	–	–	0.028	0.587
Incidence of diarrhea (d 43 to d 56), %	–	–	–	–	NS	NS

^{a,b}The values in the same row with different superscripts are significantly different ($p < 0.05$), while values with the same or no superscripts mean no significant difference ($p > 0.05$).
[1]Treatments: C, no supplementation (control); T1 = 0.5 g/calf/day; T2 = 1 g/calf/day; T3 = 2 g/calf/day.
ADG, average daily gain; SEM, standard error of mean.

TABLE 3 | The serum immunoglobulin concentrations in Holstein dairy calves fed with different doses of multispecies probiotics.

Items	Treatment (Trt)[1]				SEM	*p*-Value					
	C	T1	T2	T3		Trt	Time	Trt × Time	C × T1	C × T2	C × T3
IgA, μg/ml											
WK2	2,608.6^{c}	2,939.47^{bc}	3,351.6^{ab}	3,699.25^{a}	150.30	<0.01	0.05	0.97	0.27	0.02	<0.01
WK4	2,822.91^{b}	3,041.56^{b}	3,445.02^{a}	3,774.15^{a}	120.81				0.21	<0.01	<0.01
WK6	3,030.28^{c}	3,258.77^{bc}	3,572.28^{ab}	3,975.74^{a}	120.78				0.254	0.02	<0.01
WK8	2,984.88^{b}	3,301.57^{ab}	3,399.9^{ab}	3,758.25^{a}	117.73				0.28	0.17	0.02
IgM, μg/ml											
WK2	1,464.23^{c}	1,719.11^{bc}	2,118.18^{a}	1,940.73^{ab}	83.99	<0.01	<0.01	0.14	0.09	<0.01	<0.01
WK4	1,775.56^{b}	2,099.36^{a}	2,126.5^{a}	2,287.51^{a}	69.91				0.05	0.03	<0.01
WK6	1,789.02^{b}	2,153.23^{ab}	1,988.26^{b}	2,529.92^{a}	96.30				0.061	0.27	<0.01
WK8	1,849.23^{c}	2,058.37^{b}	2,420^{a}	2,287.9^{a}	70.53				0.03	<0.01	<0.01
IgG, mg/ml											
WK2	8.72	10.89	12.93	11.48	0.87	<0.01	<0.01	0.21	0.40	0.12	0.29
WK4	9.08^{b}	13.52^{ab}	13.71^{ab}	16.92^{a}	1.07				0.08	0.07	<0.01
WK6	12.16^{b}	12.96^{b}	13.81^{b}	18.51^{a}	0.92				0.67	0.39	<0.01
WK8	14.3^{b}	13.93^{b}	15.05^{b}	17.38^{a}	0.49				0.70	0.44	0.01

$^{a-c}$The values in the same row with different superscripts are significantly different ($p < 0.05$), while values with the same or no superscripts mean no significant difference ($p > 0.05$).
[1]Treatments: C, no supplementation (control); T1 = 0.5 g/calf/day; T2 = 1 g/calf/day; T3 = 2 g/calf/day.
IgA, immunoglobulin A; IgM, immunoglobulin M; IgG, immunoglobulin G; SEM, standard error of the mean.

The indicators of α diversity of bacteria showed that group T3 had a higher number of observed species and Shannon estimator than the control, T1, or T2 groups on WK2 ($p \leq 0.05$, **Table 4**). The indicators of α diversity of fungi showed that group T3 had a higher number of ACE estimator than the control, T1, and T2 groups at WK2 ($p \leq 0.05$, **Table 5**). In addition, time, and the interaction of treatment and time, had a significant effect on the α diversity of bacteria, whereas time had a significant effect on the α diversity of fungi. In addition, time, and the interaction of treatment and time, had a significant effect on α diversity of bacteria and fungi, but no differences were identified with respect to the treatment and time having a significant effect on the α diversity of fungi.

A plot of the principal coordinate analysis scores showed great similarity between group 4 of fecal microbiota at WK4, WK6, and WK8 (**Figures 1**, **2**). The diversity of the microbiota in group T1 was similar to that in the control group at WK4, WK6, and WK8, whereas that of group T3 demonstrated a marked shift along principal component 1 when compared with the control group (**Figures 1**, **2**).

The bacterial communities in the feces samples of calves at different weeks showed that the bacterial communities in WK2 (Bray–Curtis analysis of similarity or ANOSIM = 0.732), WK6 (Bray–Curtis ANOSIM = 0.526), and WK8 (Bray–Curtis ANOSIM = 0.373) were clustered. The clustering of fungi communities in WK2 (Bray–Curtis ANOSIM = 0.265),

TABLE 4 | The abundance and diversity of microbial microbiota in Holstein dairy calves fed with different doses of multispecies probiotics.

Items	Treatment (Trt)[1]				SEM[2]	*p*-Value					
	C	T1	T2	T3		Trt	Time	Trt × Time	C × T1	C × T2	C × T3
Bacteria											
Observed species											
WK2	105.00	140.25	124.75	153.50	8.6	<0.01	<0.01	<0.01	0.57	0.23	0.05
WK4	309.75	356.75	347.00	324.75	11.24				0.17	0.27	0.65
WK6	362.25^{ab}	378^{ab}	322.5^{b}	404.25^{a}	12.65				0.63	0.236	0.21
WK8	369.25^{a}	414^{a}	413.5^{a}	170^{b}	28.19				0.21	0.22	<0.01
Chao1 index											
WK2	194.80	203.37	201.63	207.16	10.72	<0.01	<0.01	<0.01	0.80	0.84	0.72
WK4	365.86	402.29	399.33	361.64	11.75				0.30	0.34	0.90
WK6	412.28^{ab}	422.08^{ab}	372.88^{b}	457.99^{a}	12.76				0.76	0.24	0.17
WK8	408.69^{a}	454.05^{a}	458.99^{a}	203.68^{b}	29.4				0.24	0.20	<0.01
Shannon index											
WK2	2.28^{ab}	1.77^{b}	2.17^{b}	2.79^{a}	0.13	<0.38	<0.01	<0.01	<0.01	0.05	0.10
WK4	3.83	3.90	3.98	3.87	0.39				0.80	0.63	0.90
WK6	3.99^{ab}	4.30^{a}	3.68^{b}	4.19^{ab}	0.1				0.26	0.24	0.46
WK8	3.94^{ab}	4.26^{a}	4.29^{a}	3.09^{b}	0.18				0.46	0.42	0.06
Simpson index											
WK2	0.19^{ab}	0.30^{a}	0.22^{ab}	0.13^{b}	0.02	<0.01	0.09	<0.01	0.01	0.13	0.33
WK4	0.05	0.07	0.04	0.06	0.01				0.47	0.85	0.77
WK6	0.06	0.03	0.07	0.04	0.008				0.19	0.61	0.36
WK8	0.06	0.04	0.04	0.15	0.02				0.71	0.70	0.20
ACE index											
WK2	186.58	217.03	200.34	245.47	12.42	0.01	<0.01	<0.01	0.40	0.70	0.12
WK4	362.02	400.26	389.64	359.31	11.04				0.25	0.40	0.93
WK6	405.99^{ab}	414.34^{ab}	367.31^{b}	452.11^{a}	12.24				0.78	0.22	0.14
WK8	411.65^{a}	452.07^{a}	455.97^{a}	195.64^{b}	29.93				0.28	0.24	<0.01
Fungi											
Observed species											
WK2	19.50^{b}	66.75^{a}	43.00^{ab}	45.00^{ab}	6.60	0.39	<0.01	<0.01	0.80	0.77	
WK4	41.25	80.50	80.75	59.25	6.98				0.04	0.04	
WK6	132.50^{a}	61.50^{b}	62.50^{b}	99.75^{ab}	10.85				0.13	0.14	
WK8	134.25	131.50	125.50	51.00	15.46				0.95	0.83	
Chao1 index											
WK2	20.50^{b}	74.58^{a}	48.10^{ab}	51.18^{ab}	7.46	0.43	<0.01	0.03	0.01	0.14	
WK4	45.64	95.51	89.68	72.67	8.04				0.06	0.05	
WK6	152.22^{a}	69.41^{b}	93.25^{ab}	109.06^{ab}	13.20				0.03	0.10	
WK8	149.94	149.61	141.48	62.92	17.67				0.99	0.86	
Shannon index											
WK2	1.03^{b}	1.85^{ab}	2.01^{a}	1.79^{ab}	0.16	0.36	0.01	0.17	0.07	0.03	
WK4	2.01^{b}	2.06^{ab}	2.53^{a}	2.44^{ab}	0.09				0.79	0.03	
WK6	2.47	2.30	2.16	1.71	0.18				0.75	0.56	
WK8	2.30	2.31	2.28	1.97	0.08				0.97	0.94	
Simpson index											
WK2	0.52	0.33	0.26	0.30	0.05	0.67	0.02	0.15	0.16	0.06	
WK4	0.26	0.23	0.16	0.16	0.02				0.52	0.05	
WK6	0.20	0.22	0.31	0.40	0.04				0.88	0.35	
WK8	0.22	0.25	0.24	0.27	0.02				0.60	0.73	
ACE index											
WK2	20.83^{b}	83.20^{a}	48.07^{ab}	56.12^{ab}	8.03	0.31	<0.01	0.03	<0.01	0.15	
WK4	45.64^{b}	95.51^{a}	89.68^{ab}	72.67^{ab}	8.04				0.028	0.047	
WK6	146.15	84.74	110.37	113.63	10.92				0.06	0.25	
WK8	149.29	152.97	142.02	63.25	17.24				0.94	0.87	

^{a,b}The values in the same row with different superscripts are significantly different ($p < 0.05$), while values with the same or no superscripts mean no significant difference ($p > 0.05$).

[1]Treatments: C, no supplementation (control); T1 = 0.5 g/calf/day; T2 = 1 g/calf/day; T3 = 2 g/calf/day.

SEM, standard error of mean.

TABLE 5 | Analysis of similarity (ANOSIM) of multispecies probiotics on fecal bacteria and fungi of Holstein dairy calves.

Variable[1]	Bray–Curtis ANOSIM	*p*-Value[2]	Binary Jaccard ANOSIM	*p*-Value[2]
Bacteria				
WK2	0.732	0.001	0.532	0.001
WK4	0.077	NS	0.027	NS
WK6	0.526	0.001	0.358	0.001
WK8	0.373	0.001	0.381	0.002
Fungi				
WK2	0.265	0.009	0.139	0.084
WK4	0.281	0.013	0.279	0.002
WK6	−0.030	NS	0.171	0.032
WK8	0.308	0.007	0.212	0.087

[1]*Directly fed compound probiotics for pre-weaning calves of different ages at WK2, WK4, WK6, and WK8.*
[2]*NS, not significant ($p > 0.05$). $1 > r > 0$, indicates a significant difference between groups; $-1 < r < 0$ indicates that the difference between groups is not significant.*

WK4 (Bray–Curtis ANOSIM = 0.281), and WK8 (Bray–Curtis ANOSIM = 0.308) indicates that the inter-group difference is more significant than the intra-group difference. There were no significant differences between the bacteria in WK4 (Bray–Curtis ANOSIM = 0.077) and fungi in WK6 (Bray–Curtis ANOSIM = −0.030). Principal coordinates analysis (PCoA) and ANOSIM analyses revealed that adding different doses of MSP during the feeding process of pre-weaning calves exert significant differences in the microbial structure of the fecal microbiota (**Table 5**).

Relative Abundance of Bacterial and Fungal Taxa

A comparison of the effects of groups T1, T2, and T3 supplementation on the fecal microbial composition of pre-weaning calves on different weeks was carried out using a taxon-dependent analysis. *Firmicutes* and *Bacteroidetes* were the dominant bacteria phyla, followed by *Actinobacteria*, *Proteobacteria*, and *Tenericutes* (**Figures 3A,B**). The relative abundance of *Firmicutes* was identified for calves of T3 group compared with the control group at WK8 ($p < 0.01$; **Supplementary Table 1**). However, as the calves grew older and treatments continued, no significant changes were observed in the relative abundance across the phyla except for *Bacteroidetes* and *Fusobacteria* (**Figures 3A,B** and **Supplementary Table 2**). *Blautia* and *Ruminococcaceae_UCG005* were the predominant bacteria genera, followed by *Ruminococcaceae_UCG-014*,

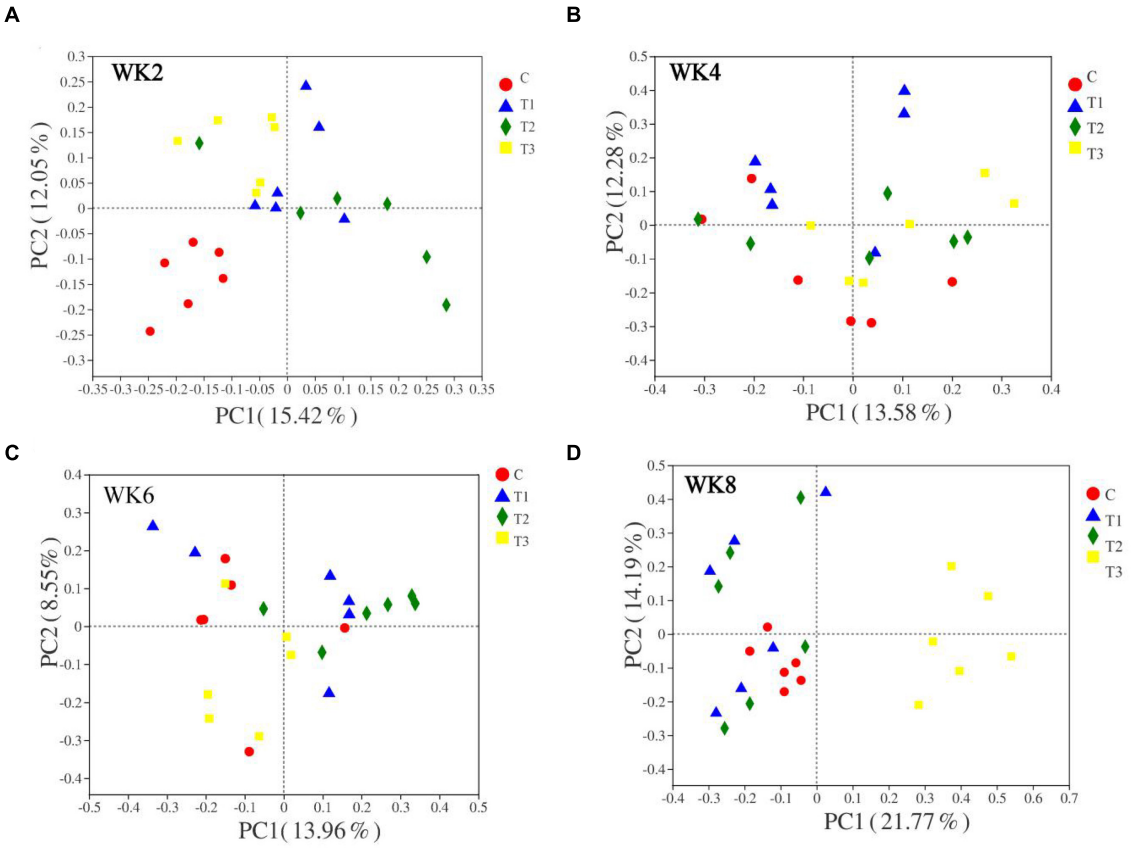

FIGURE 1 | Based on Bray–Curtis distance principal coordinates analysis (PCoA) (Panels **A–D** indicate WK2, WK4, WK6, and WK8 bacteria PCoA pictures).

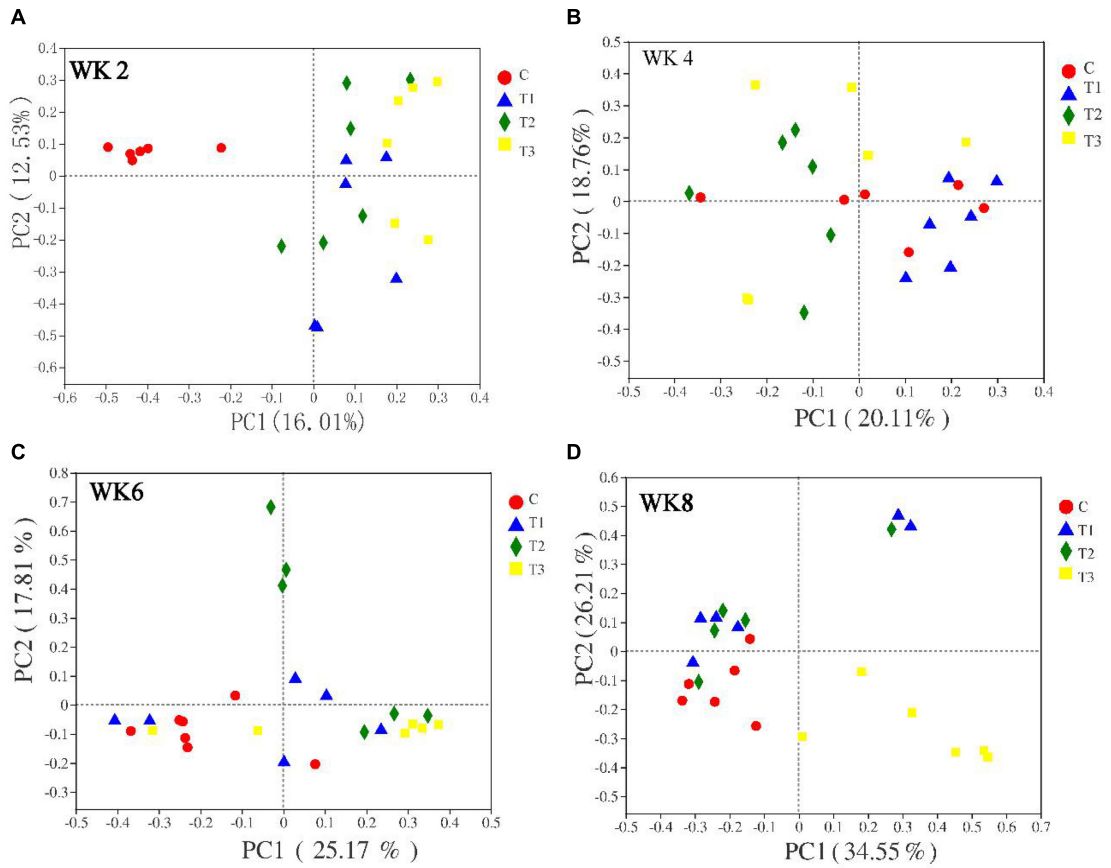

FIGURE 2 | Based on Bray–Curtis distance principal coordinates analysis (PCoA) (Panels **A–D** indicate WK2, WK4, WK6, and WK8 fungi PCoA pictures).

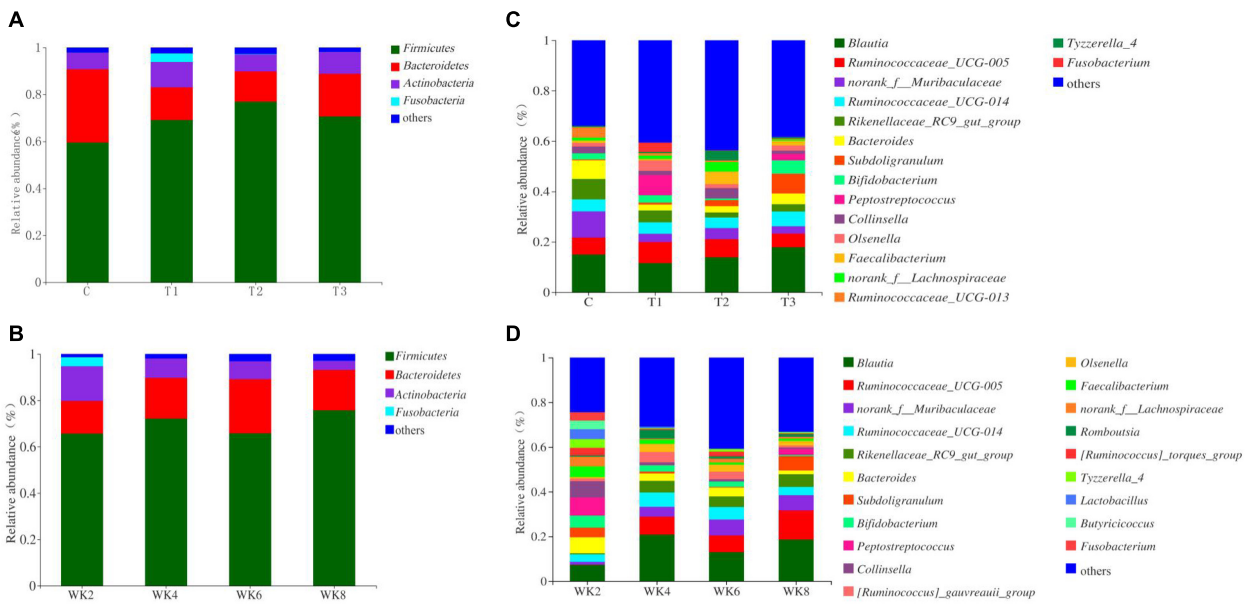

FIGURE 3 | Composition of the fecal microbial community. Panels **(A,B)** show the composition of the microbial community in the four groups (C, T1, T2, and T3) with respect to the phyla and genera of bacterial. Panels **(C,D)** show the compositions at four time points during the study (WK 2, 4, 6, and 8).

Rikenellaceae_RC9_gut_group, *Bacteroides*, *Subdoligranulum*, *Bifidobacterium*, *Peptostreptococcus*, *Collinsella*, *Lactobacillus*, *Butyricicoccus*, and *Dorea* (**Figures 3C,D**). The relative abundances of *Bifidobacterium* and *Ruminococcaceae_UCG-014* tended to be higher in group T3 than in the control, T1, and T2 groups at WK2 ($p = 0.05$, $p = 0.03$, respectively; **Supplementary Table 2**). In addition, time had no significant effect on the representation of genera except for *Blautia*, *Collinsella*, *Lactobacillus*, *Butyricicoccus*, and *Dorea* ($p = 0.03$, $p < 0.01$, $P < 0.01$, $P = 0.01$, $P < 0.01$, respectively). Treatment and treatment × time had no significant effect on the representation of genera except for the *Ruminococcaceae_UCG005*, *norank_f__Muribaculaceae*, *Rikenellaceae_RC9_gut_group*, *Peptostreptococcus*, or *[Ruminococcus]_gauvreauii_group* genera ($P < 0.01$, respectively).

Ascomycota and *Basidiomycota* were the dominant fungal phyla in calf rectal microbial composition (**Figures 4A,B**), followed by *Neocallimastigomycota* and unclassified fungi. However, as the calves grew older, time and treatment had no significant effect on the relative abundance of fungal phyla. *Aspergillus* and *Thermomyces* were the predominant fungal genera, followed by *Saccharomyces, Melanocarpus*, *Cutaneotrichosporon*, *Pichia*, *Wallemia*, *Chrysosporium*, *Acrostalagmus*, *Microascus*, *Nigrospora*, and *Kazachstania* (**Figures 4C,D**). The relative abundances of *Aspergillus*, *Saccharomyces*, *Melanocarpus*, and *Chrysosporium* tended to be higher in the T3 group than that in the control, T1, and T2 group at WK2 ($p = 0.03$; $p = 0.01$; $p = 0.05$; $p = 0.03$, respectively; **Supplementary Table 2**). The relative abundances of *Pichia* tended to be lower in the group T3 than that in the control group at WK2 ($p < 0.05$, **Supplementary Table 2**). In addition, time had no significant effect on the representation of each genera except for the relative abundance of *Microascus* and *unclassified_k__Fungi* ($p < 0.01$; $p = 0.02$; respectively). Time and trt-time had no significant effect on the representation across genera except for *Cutaneotrichosporon* ($p = 0.02$, $p = 0.03$, respectively).

Relationships Between the Size of Microbial Populations and Immunoglobulin Indices

We next analyzed the relationship between the size of each fecal microbial population at WK2, and both growth performance and immunoglobulin indices using Pearson's rank correlation coefficients. At the genus level, the relative abundance of *Bifidobacterium* positively correlated with ADG concentrations ($p < 0.05$; **Table 6**), while the relative abundance of *Melanocarpus* positively correlated with total feed intake and starter intake ($p < 0.05$), and the relative abundance of *Saccharomyces* positively correlated with IgA and IgG ($p < 0.05$).

The relative abundance of *Blautia* correlated negatively with total feed intake, starter intake, and IgG ($p < 0.05$). The relative abundance of *Collinsella* correlated negatively with

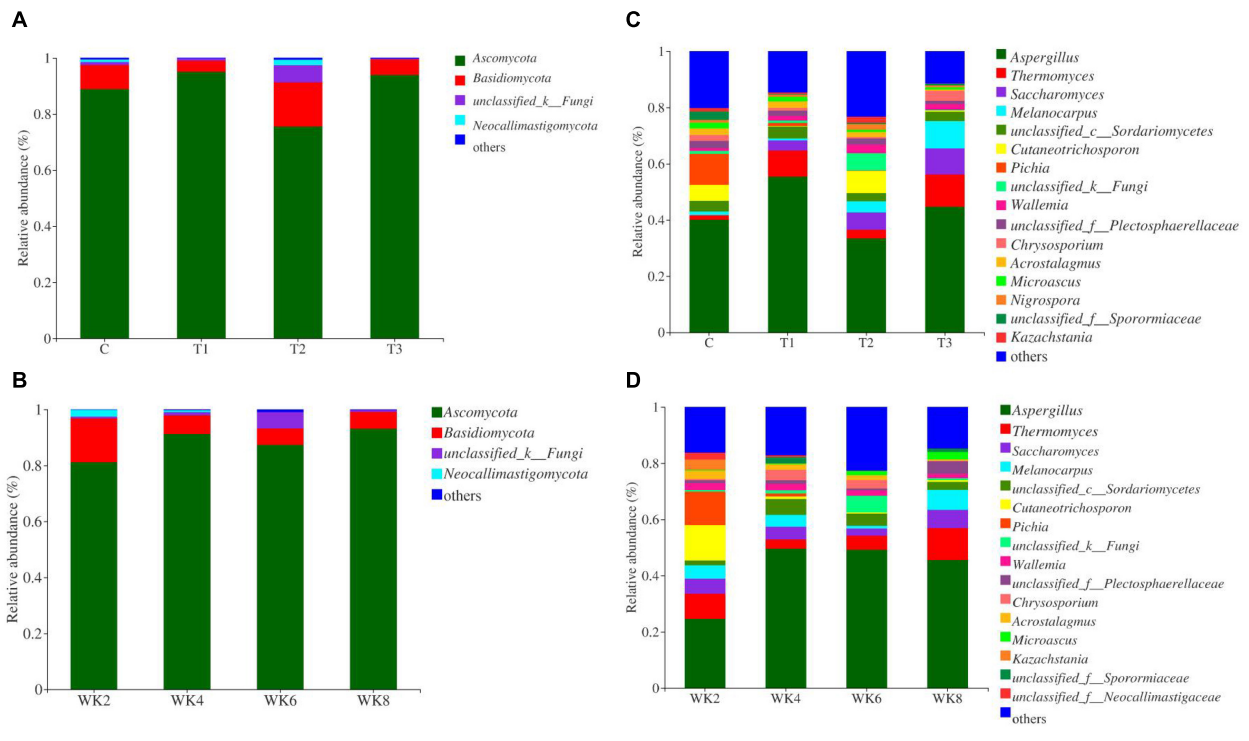

FIGURE 4 | Composition of the fecal microbial community. Panels **(A,B)** show the composition of the microbial community in the four groups (C, T1, T2, and T3) with respect to the phyla and genera of fungi. Panels **(C,D)** show the compositions at four time points during the study (WK 2, 4, 6, and 8).

TABLE 6 | Pearson correlation coefficients between the populations of the principal genera at WK 2 and immunoglobulin indices.

Genus	ADG	Total feed intake	Starter intake	Feed efficiency	Serum concentration		
					IgA	IgG	IgM
Bacteria							
Blautia	0.18	−0.71*	−0.71*	0.21	−0.35	−0.57*	−0.39
Bacteroides	0.20	−0.37	−0.37	0.36	−0.72*	−0.28	−0.37
Ruminococcaceae_UCG-014	0.43	0.28	0.28	0.14	0.37	0.19	0.26
Peptostreptococcus	0.07	0.37	0.37	−0.29	−0.15	0.05	−0.12
Bifidobacterium	0.34*	0.05	0.05	0.32	0.38	0.00	0.09
Collinsella	−0.54*	−0.25	−0.25	−0.26	−0.18	−0.02	0.09
Faecalibacterium	−0.07	0.00	0.00	0.21	0.33	0.00	0.24
Subdoligranulum	−0.04	0.02	0.02	0.13	0.37	0.22	0.40
Lactobacillus	−0.15	0.16	0.16	0.12	0.01	0.25	0.10
Norank_f__Lachnospiraceae	−0.28	−0.09	−0.09	−0.58	−0.23	0.28	0.30
Tyzzerella_4	−0.41	−0.02	−0.02	−0.03	0.24	0.39	0.40
Fusobacterium	0.01	0.06	0.06	−0.14	−0.21	−0.18	−0.21
Butyricicoccus	−0.34	−0.38	−0.38	−0.20	−0.29	−0.39	−0.42
[Ruminococcus]_torques_group	−0.49	−0.05	−0.05	−0.19	−0.21	−0.07	−0.02
Dorea	−0.36	−0.05	−0.05	0.18	0.33	0.46	0.44
Corynebacterium_1	0.18	0.05	0.05	−0.04	0.12	0.02	−0.10
Peptoclostridium	−0.08	−0.04	−0.04	−0.04	0.18	0.12	0.21
Prevotella_7	0.24	0.17	0.17	0.22	0.38	0.14	0.25
Norank_f__Muribaculaceae	0.27	0.19	0.19	0.36	0.13	−0.02	−0.33
Peptostreptococcus	0.07	0.37	0.37	−0.29	−0.15	0.05	−0.12
Fungi							
Aspergillus	0.23	0.38	0.38	0.04	0.28	0.00	0.22
Cutaneotrichosporon	−0.29	−0.60**	−0.60**	−0.22	−0.30	−0.24	0.11
Pichia	0.15	−0.21	−0.21	−0.65	−0.55*	−0.40	−0.45
Thermomyces	0.09	0.50	0.50	0.02	0.23	0.23	0.26
Saccharomyces	−0.15	0.23	0.23	0.05	0.52*	0.56*	0.73
Melanocarpus	0.30	0.51*	0.51*	0.24	0.45	0.24	0.26
Kazachstania	−0.27	0.21	0.21	−0.14	0.01	0.47	0.09
Acrostalagmus	0.23	0.05	0.05	−0.06	−0.24	−0.03	−0.12
Unclassified_f__Neocallimastigaceae	−0.18	0.18	0.18	0.09	0.05	0.39	0.02
Wallemia	−0.04	0.11	0.11	0.06	0.40	0.11	0.45
Unclassified_o__Chaetothyriales	−0.07	−0.05	−0.05	0.04	−0.35*	−0.01	−0.53
Unclassified_c__Sordariomycetes	0.05	0.27	0.27	0.12	0.51	0.42	0.57
Unclassified_p__Ascomycota	−0.40	0.14	0.14	−0.29	−0.15	0.34	−0.08
Candida	0.42	−0.05	−0.05	−0.21	−0.43	−0.29	−0.42
Ramularia	−0.37	0.25	0.25	0.28	−0.08	0.36	−0.31
Unclassified_f__Plectosphaerellaceae	−08	0.04	0.04	0.33	0.46	0.30	0.59
Veronaea	−0.09	−0.14	−0.14	0.17	0.25	0.08	0.25
Paraphaeosphaeria	−0.30	0.12	0.12	−0.23	−0.27	0.21	−0.12
Acremonium	−0.20	0.23	0.23	0.25	0.24	0.28	−0.15
Unclassified_k__Fungi	−0.14	0.15	0.15	−0.05	−0.24	−0.08	0.08
Nigrospora	−0.14	0.21	0.21	−0.12	0.22	0.38	0.41
Unclassified_o__Pleosporales	0.18	0.14	0.14	0.11	0.28	0.16	0.22
Golovinomyces	−0.26	−0.25	−0.25	−0.42	−0.14	0.31	0.36
Chrysosporium	0.39	0.41	0.41	0.26	0.28	0.00	0.22

*$p < 0.05$, **$p < 0.01$.

ADG concentrations ($p < 0.05$). The relative abundance of *Bacteroides* also showed a negative correlation with IgA ($p < 0.05$). A negative correlation was also found between the relative abundance of *Cutaneotrichosporon* and that of feed intake and starter intake concentrations ($p < 0.01$).

DISCUSSION

The gut microbial colonization of ruminants gradually colonizes from the fetal period to after birth (Klein-Jöbstl et al., 2019; Bi et al., 2021). Early gut microbiota plays a vital role in the long-term health of the host (Malmuthuge and Guan, 2017).

The intestinal microbiota of newborn calves changes during the early postnatal period (Malmuthuge et al., 2015; Takino et al., 2017; Song et al., 2018; Klein-Jöbstl et al., 2019). Therefore, the probiotic supplementation provides opportunities to improve early-life gut health and to minimize calves' susceptibility to enteric infections during the pre-weaning period (Van den Abbeele et al., 2011; Malmuthuge and Guan, 2017; Markowiak and Śliżewska, 2018).

The present study showed that supplementation with MSP in group T3, but not groups T1 and T2, significantly increased the ADG and total feed intake of newborn calves in the first 8 weeks after birth. *Lactobacillus acidophilus* (Bayatkouhsar et al., 2013; Foditsch et al., 2015; Sharma et al., 2018), *Bacillus subtilis* (Sun et al., 2010; Zhang et al., 2017), and *Saccharomyces cerevisiae* (Villot et al., 2019) had growth-promoting effects. Timmerman et al. (2005) also found that MSP-treated veal calves had growth-promoting effects over placebo-treated veal calves from day 1 to 56, but the results were not statistically significant. In the present study, calves gained 875 and 720 g/day of growth in the T1 and T3 group, respectively. This result is consistent with the findings of Renaud et al. (2019), who showed that calves gained 630 g/day of growth when receiving a 4-g bolus of the MSP. Notably, the total feed intake in the T3 group tended to be higher than in the T1, T2, and control groups, which might be due to the higher bioavailability of MSP that can produce organic acids and many kinds of metabolites in the process of animal metabolism enzymes and some important nutrients (Pandey et al., 2015). Differences in results between the different tests may be related to the type, quantity, proportion, and method of probiotic delivery, as well as the different management levels between the cattle farms.

In the present study, the incidence of diarrhea in control calves fluctuated between 1.14% and 25.11% during the first 4 weeks of life. However, supplementation with MSP was helpful to reduce the incidence of diarrhea in neonatal dairy calves during day 7–21 after birth (Ma et al., 2020), which is consistent with previous studies (Wehnes et al., 2009; Novak et al., 2012; Renaud et al., 2019). Prevention and control of outbreaks before occurring are more cost effective (Knights et al., 2011), and current studies have found that early intervention of probiotics has a better preventive effect (Hempel et al., 2012; Guarino et al., 2015; Hua et al., 2016). Malmuthuge and Griebel (2018) and other researchers have described the potential strategies for controlling early microbiota and to improve the health of newborn calves during the period when they are most susceptible to intestinal diseases. We found that compared with the control group, groups T1, T2, and T3 significantly reduced the diarrheal rates of calves that were 4 weeks old or less, but had no significant effect on those that were between WK6 and WK8 old. However, there is limited information on specific changes in fecal microbiota resulting from the direct feeding of multispecies probiotics to neonatal calves.

One of the recent studies have proposed that supplementation with *Saccharomyces cerevisiae* increased the immune responsiveness of calves by increasing IgA concentration (Villot et al., 2020). Furthermore, Sun et al. (2020) showed that *Bacillus subtilis natto* increased general performance by improving the ADG and feed efficiency, and advanced the weaning age of the calves. While there is no difference in serum IgA and IgM, serum IgG was higher in the *Bacillus subtilis natto*-supplemented calves than in the control calves. Consistent with these findings, we found that MSP supplementation in the T3 group increased serum IgA, IgM, and IgG concentrations above those of the control by 1.1, 0.48, and 2.76 mg/ml, respectively, compared with groups T1 and T2 supplementation, indicating that group T3 is superior to groups T1 and T2 with respect to the immune function of dairy calves.

We observed no significant difference in the Shannon index between WK2 and WK8 after adding multispecies probiotics. This finding is similar to the change in fecal microbiota in the first 8 weeks of calves reported earlier (Knights et al., 2011; Cho and Yoon, 2014). However, adding probiotics to the calves' diet before weaning can change the bacterial diversity and composition of the gastrointestinal tract, but has little impact on the diversity and a greater impact on the composition of the microbial community (Villot et al., 2019). After adding MSP, we compared the outcomes with the control group. In WK2, the MSP supplementation increased the relative abundance of *Firmicutes* and significantly reduced the relative abundance of *Bacteroidetes*. *Firmicutes*, *Bacteroidetes,* and *Actinobacteria* are the dominant microbial taxa in the hindgut of pre-weaning calves and humans (Song et al., 2018; Kassaian et al., 2020). *Firmicutes* is often the dominant phylum in most animal species (Guarino et al., 2015). In diarrheic intestines of children, *Bacteroides* remain the dominant genera. In this study, probiotics significantly reduced the relative abundance of *Bacteroides* while increasing the relative abundance of *Ruminococcaceae_UCG-005* (Saraf et al., 2017).

The representative genera from *Bifidobacterium*, *Lactobacillus*, *Subdoligranulum*, *Blautia*, and *Bacteroides* were closely related to healthy calves (Jang et al., 2019; Schwaiger et al., 2020), which is consistent with the findings of our study. The presence of *Bifidobacterium* family D7 at birth is similar to the fecal microbiota of vaginal delivery in infants (Kassaian et al., 2020) and the intestinal tract of early infants (Arrieta et al., 2014). In this study, the abundance of *Bifidobacterium* was higher at WK2 than in the older calves (WK4, WK6, and WK8). *Bifidobacteria* plays an important role in immune stimulation in host invasion (Hidalgo-Cantabrana et al., 2014). Additionally, the gene expression and microRNA expression in the small intestine of the same calf were highly correlated to the number of *Bifidobacteria* (Liang et al., 2014). Therefore, it is important to know how the diversity of *Bifidobacterium* could be impacted by age and how this influences host functions. The differences in the composition of intestinal microbiota may not be the cause of diarrhea as some changes in bacterial abundance may guide our interpretation of diarrhea. This study provides a theoretical basis for the establishment of a control system for calf diarrhea (Arrieta et al., 2014).

The lower relative abundance of *Blautia* in diarrhea from 14-day-old calves when compared with healthy calves suggests that this genus may be associated with diarrhea (Ma et al., 2020). A high prevalence of *Blautia* has also been reported in the colon and feces of healthy neonatal swine (Saraf et al., 2017) and human infants (Jost et al., 2014;

Sagheddu et al., 2017). However, fecal samples of dogs with diarrhea showed a general reduction of *Blautia*. *Blautia* utilizes polysaccharides that cannot be used by other intestinal microorganisms to degrade and produce butyrate, which is one of the main short-chain fatty acids that maintains intestinal health and the intestinal epithelial barrier by regulating the immune system (Guarino et al., 2015). *Collinsella* can metabolize carbohydrates of plant or animal origin, and together with *Bifidobacterium*, can modify the bile acids of the host, modulating the virulence and pathogenicity of enteric pathogens (Bag et al., 2017). The relative abundances of these bacteria in this study were observed to be higher in calves supplemented with MSP at WK2. Yet, the reduction in the abundance of this genus was reported in fecal samples of dogs with diarrhea.

The abundance of *Cutaneotrichosporon* observed in the control group was significantly lower than that observed in group T3. *Cutaneotrichosporon* have been described as a lipolytic yeast species from food and food-related environments (Péter et al., 2019). *Cutaneotrichosporon debeurmannianum* is a rarely isolated yeast from human blood and urine samples, with clinical samples coming from patients that were diagnosed with septicemia and urinary tract infections. The pathogenic potential and epidemiological relevance of this yeast remains to be seen (do Espírito Santo et al., 2020). In this study, the reduced abundance of *Cutaneotrichosporon* in group T3 did lead to an increase in total feed and starter intake than that of the control group. This suggests that *Cutaneotrichosporon* in calves influences the total feed intake and starter intake, but weirdly affects other aspects as they too have also been found to cause the occurrence of diarrhea.

The abundance of *Saccharomyces* observed in group T3 was significantly higher than that observed in the control group. *Saccharomyces* is rich in digestible proteins, vitamins (vitamin B6, thiamin, biotin, riboflavin, nicotinic acid, and pantothenic acid), magnesium, and zinc (Massé and Weiser, 1994). *Saccharomyces cerevisiae* flows along the gastrointestinal tract without adhering to its walls. The strains that do not have the ability to adhere to the intestinal epithelium, which are effective as bioregulators and their action are based on the ability of colonization through several mechanisms (Rodrigues et al., 2000; Baptista, 2002). In their study, the use of live yeast cells is to act as detoxification agents against mycotoxins, and other bacterial toxins and their receptors in the mucous membrane, and *Vibrio cholerae* toxin. Severe damage to organs has been eliminated due to diets that may contain these toxins in the presence of *S. cerevisiae* for their ability to reduce animal stress, providing vitamins, enzymes, and proteins (Baptista et al., 2005). *Saccharomyces cerevisiae* in calf diets augment immunological effect by increasing IgM and IgA activity against pathogens, enhancing intestinal development and function, adsorb mycotoxins, modulate gut microbiota, and reduce post-weaning diarrhea (Qamar et al., 2001; Sun et al., 2019; Elghandour et al., 2020). We found that the increased abundance of *Saccharomyces* in group T3 improved the concentrations of IgA and IgG than the control group. This result further supports the hypothesis that adding MSP may affect overall IgA and IgG serum concentrations by influencing the fecal microbial composition in calves.

CONCLUSION

We demonstrated that supplementation with MSP in the T1, T2, and T3 groups had more advantages than the control group in terms of promoting growth performance and reducing the incidence of diarrhea in pre-weaning dairy calves. However, no significant differences were identified between the MSP and control groups with respect to these end points. The dose of MSP supplementation in group T3 had more advantages in reducing the incidence of diarrhea from WK2 to WK4 in newborn calves than groups T1, T2, and the control. Supplementation with MSP increased serum IgA and IgM concentrations in group T3 to levels that were significantly higher than those in the control group between WK2 and WK8. This implies that group T3 has relatively higher bioavailability than groups T1 and T2. Therefore, it indicated that the lower incidence of diarrhea in calves receiving MSP supplement is the result of an effect on fecal microbial composition and diversity. In view of their differing effects, we recommend adding 2 g/day of MSP supplementation in diets of dairy calves during their first 4 weeks of life before weaning. Our findings provide a basis for the rational use of MSP supplementation in calf production and may help to reduce the use of antibacterial agents.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of the College of Animal Science and Technology, Shihezi University (No. A2019-155-01).

AUTHOR CONTRIBUTIONS

YW and WZ carried out the experimental design of this study. YW, RL, and HC contributed to the experimental implementation. YW and XL contributed to sampling of this study. YW and CN contributed to the data analysis. YW, LW, JN, CC, and YX contributed to the article writing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.681014/full#supplementary-material

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REVIEW
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The Microbiota-Gut-Brain Axis in Health and Disease and Its Implications for Translational Research

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Over the past decades, microbiome research has evolved rapidly and became a hot topic in basic, preclinical and clinical research, for the pharmaceutical industry and for the general public. With the help of new high-throughput sequencing technologies tremendous progress has been made in the characterization of host-microbiota interactions identifying the microbiome as a major factor shaping mammalian physiology. This development also led to the discovery of the gut-brain axis as the crucial connection between gut microbiota and the nervous system. Consequently, a rapidly growing body of evidence emerged suggesting that the commensal gut microbiota plays a vital role in brain physiology. Moreover, it became evident that the communication along this microbiota-gut-brain axis is bidirectional and primarily mediated by biologically active microbial molecules and metabolites. Further, intestinal dysbiosis leading to changes in the bidirectional relationship between gut microbiota and the nervous system was linked to the pathogenesis of several psychiatric and neurological disorders. Here, we discuss the impact of the gut microbiota on the brain in health and disease, specifically as regards to neuronal homeostasis, development and normal aging as well as their role in neurological diseases of the highest socioeconomic burden such as Alzheimer's disease and stroke. Subsequently, we utilize Alzheimer's disease and stroke to examine the translational research value of current mouse models in the spotlight of microbiome research. Finally, we propose future strategies on how we could conduct translational microbiome research in the field of neuroscience that may lead to the identification of novel treatments for human diseases.

Keywords: gut microbiota, gut-brain axis, germ-free rodent, short-chain fatty acids, wildling, microglia, Alzheimer's disease, stroke

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Abbreviations: AD, Alzheimer's disease; AGEs, advanced glycation end products; APP, amyloid precursor protein; ASD, autism spectrum disorder; BBB, blood–brain barrier; BDNF, brain-derived neurotrophic factor; CITE-seq, cellular indexing of transcriptomes and epitopes by sequencing; CNS, central nervous system; CPT1, carnitine palmitoyltransferase 1; FMT, fecal microbiota transplantation; GF, germ-free; ILCs, innate lymphoid cells; MAMPs, microbial-associated molecular patterns; MCA, middle cerebral artery; Mn, manganese; MS, multiple sclerosis; PD, Parkinson's disease; PFC, pre-frontal cortex; PS1, presenilin 1; RAGE, receptor for AGEs; SCFAs, short-chain fatty acids; SPF, specific-pathogen-free; Th1, T helper 1; TMAO, trimethylamine N-oxide.

INTRODUCTION

All mammals are multicellular organisms that are not only composed of their own individual cells but also of the microorganisms that inhabit all epithelial barrier sites and well-defined, distinct ecological niches such as the gastrointestinal tract, the skin, the respiratory tract, and the genitourinary system. In this context, it was shown that not just the number of microbial cells in the body (Sender et al., 2016) but also their genetic material vastly exceeds the corresponding human counterparts which emphasizes the potential of the microbiota to influence the host (Li et al., 2014; Xie et al., 2016; Lloyd-Price et al., 2017). Microbiota is defined as all microbes present within an ecological niche such as the gut, whereas the microbiome is the combination of the microbiota and their genes (Berg et al., 2020). By now we know that the vast majority of microorganisms inhabit the gastrointestinal tract (Berg, 1996). The gut microbiota is a complex and diverse ecosystem comprising microbes of all kingdoms such as bacteria, archaea, viruses, fungi, protozoa, and the meiofauna. It is acquired through vertical transmission and environmental exposure. Importantly, these microbes do not just passively colonize the gut of their hosts, in contrast, they established an intimate, mutually beneficial, symbiotic relationship over eons of co-evolution. The host provides its microbial subtenants with a habitat as well as nutrients, and in return, the microbes offer various health benefits (Dethlefsen et al., 2007; Ley et al., 2008; Sommer and Bäckhed, 2013). For instance, the commensal gut microbiota plays an essential role for the synthesis of several vitamins (e.g., vitamins K and B), it provides energy for the host in terms of short-chain fatty acids (SCFAs, e.g., butyrate) by fermentation of otherwise indigestible carbohydrates as well as fibers and it is involved in the metabolism of bile acids, sterols and the deactivation of xenobiotics (Cummings and Macfarlane, 1997; O'Hara and Shanahan, 2006). Taken together, the gut microbiome plays a vital role in virtually every aspect of mammalian physiology, specifically in the development, maturation, homeostasis, and ultimately the function of the immune system in health and disease (Cerf-Bensussan and Gaboriau-Routhiau, 2010; Hooper et al., 2012; Belkaid and Hand, 2014; Zheng et al., 2020). Hence, the mammalian phenotype is driven by a combination of the host genome and the microbial genome aka microbiome, together referred to as the metagenome, which is also acknowledged in the holobiont theory or the meta-organism concept (Norman et al., 2014; Stappenbeck and Virgin, 2016).

The source of this increasing understanding regarding host-microbiota interactions certainly was the development of high-throughput sequencing technologies, which enabled scientists to perform metagenomic studies (Arnold et al., 2016). Naturally, there was also growing evidence of a bidirectional communication between the central nervous system (CNS) and the gastrointestinal microbiota called the microbiota-gut-brain axis, which turned out to be an essential contributor to overall brain physiology. There are complex and manifold ways the microbiota uses to communicate and influence the host via this axis (Collins et al., 2012). Microbiota can have a direct impact on the production of metabolic precursors like tryptophan or the synthesis of neurotransmitters like serotonin and dopamine or they produce SCFAs (Diaz Heijtz et al., 2011; Sampson and Mazmanian, 2015; Yano et al., 2015). SCFAs can also act in an epigenetic fashion by inhibiting histone deacetylases (Wu et al., 2012). Further, the microbiota and the CNS are linked by the release of microbial-associated molecular patterns (MAMPs) (Sampson and Mazmanian, 2015). MAMPs include bacterial derived molecules like double-stranded RNA, lipopolysaccharide and lipoproteins which are recognized by different receptors, mainly belonging to Toll-like receptors (Akira and Hemmi, 2003). Additionally, the gut is connected to the CNS through the vagus nerve and enables a direct communication through neurochemicals (Forsythe et al., 2014). Jonathan Kipnis even proposed a defining role of the immune system to sense microorganisms and to deliver relevant information about them to the brain via different immune signaling molecules like cytokines (Kipnis, 2018). However, bio-active microbial molecules and metabolites appear to play a dominant role in mediating the communication along the gut-brain axis.

Animal models were crucial in highlighting and understanding the manifold impact of microbes on the nervous system in development, maturation, aging and homeostasis such as changes in expression of neurotrophic factors, NMDA receptor subunits in the hippocampus (Sudo et al., 2004; Bercik et al., 2011; Diaz Heijtz et al., 2011), impaired blood-brain barrier function, increased myelination in the prefrontal cortex (Braniste et al., 2014; Hoban et al., 2016) as well as learning and memory (Gareau et al., 2011; Neufeld et al., 2011; Clarke et al., 2013; Swann et al., 2020). Further, there is also some evidence for a role of microbiota in psychiatric disorders like depression and anxiety (Foster and McVey Neufeld, 2013), autism spectrum disorder (ASD) (Krajmalnik-Brown et al., 2015; Marrone and Coccurello, 2019), schizophrenia (Severance et al., 2016; Marrone and Coccurello, 2019) as well as neurological diseases such as Alzheimer's disease (AD) (Kowalski and Mulak, 2019), Parkinson's disease (PD) (Keshavarzian et al., 2015) and stroke (Durgan et al., 2019; Battaglini et al., 2020). Additionally, gut microbiota has been suggested to control the maturation and function of microglia, the CNS resident immune cells (Erny et al., 2015; Matcovitch-Natan et al., 2016; Louveau and Kipnis, 2018; Thion et al., 2018). Since microglia lie at the interface between environmental signals and the brain, they may be a critical link between the microbiome and the CNS. Actually, microglia has been shown to contribute to CNS diseases such as ASD, AD, multiple sclerosis (MS), and PD, all of which are also affected by gut microbiota (Sharon et al., 2016).

Thus, conventional laboratory mice, especially germ-free (GF), antibiotic-treated, gnotobiotic and specific-pathogen-free (SPF) mice were an invaluable tool for proof-of-principal studies that highlighted the profound impact of microbiota on mammalian physiology. Through mouse models, scientist learned about the importance of microbiota in health and disease, thereby identifying microbiota as a potential target for novel treatment strategies above and beyond the field of neuroscience. However, aside from these valuable proof-of-principal studies, there are only few success stories where basic biological principals discovered in mice could be directly translated into the human

system, creating breakthroughs in translational medicine such as immune checkpoint inhibitors (Littman, 2015) as well as the discovery of MHC restriction (Zinkernagel and Doherty, 1974a,b). In general, laboratory mouse models appear to be fairly limited when it comes to predicting complex physiological responses of humans. Hence, the transition from preclinical studies in mice to bedside practice in humans rarely works (Mestas and Hughes, 2004; von Herrath and Nepom, 2005; Payne and Crooks, 2007; Seok et al., 2013; Shay et al., 2013; Hay et al., 2014; Mak et al., 2014), especially in neurosciences (Garner, 2014; Pound and Rebecca, 2020). There is a vast body of literature illustrating that the conventional laboratory mouse has not only misdirected clinical approaches consuming trillions of research funding (Wong et al., 2019), but also let to catastrophically failed human clinical trials (Fisher et al., 1996; Suntharalingam et al., 2006). The consequent and crucial question is how to improve the translational research value of animal models and increase the safety and success rate of transitioning treatments into the clinic?

Here, we want to highlight how the gut microbiota impacts the development, maturation, aging, homeostasis and function of the brain. Further, we show how microbiota potentiates disease states in AD and stroke in a negative as well as positive way. Subsequently, we exemplify the translational research value of the laboratory mouse in the context of AD and stroke research. Finally, we speculate on how the recent progress in the development of translational research models may be utilized in the field of translational microbiome neuroscience to maximize the clinical benefit and identification of novel treatments.

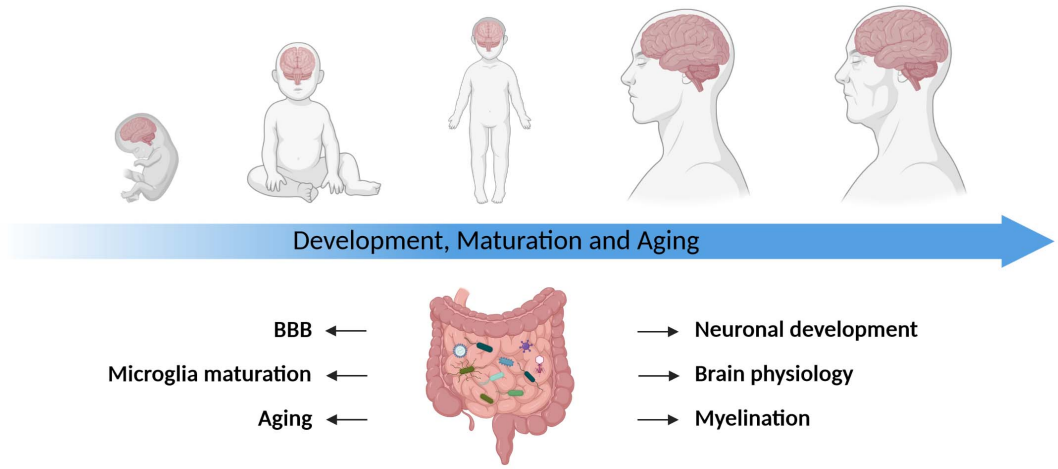
THE INFLUENCE OF THE GUT MICROBIOME ON THE DEVELOPMENT OF THE NERVOUS SYSTEM

The microbial colonization of all mammalian organisms starts at birth with the passage of the newborns through the birth canal and the exposure to the vaginal microbiome of their mothers. Subsequently, the first contact between the immune system and the microbiota happens at all epithelial barrier sites of the body, thereby shaping immune tolerance to commensal microbes and establishing barrier integrity. The infant's microbiome develops and stabilizes within 2–3 years in humans (Stecher, 2015) and by the age of 2–3 weeks in mice (Schloss et al., 2012). Importantly, this early window of life is not only characterized by critical phases of development and growth involving barrier sites themselves, but also involves virtually all organs distal to barrier sites and multi-organ structures such as the immune system (Arrieta et al., 2014). Remarkably, the brain increases its size more than 50% during the first 3 months of life and reaches 90% of the size of an adult organ within the first 5 years (Tierney and Nelson, 2009). The vast majority of neuronal development happens during this pivotal phase (O'Mahony et al., 2017). Since the periods of microbiota development coincides with the majority of brain development, researchers hypothesized early on that these times of parallel development may be of high biological relevance. This was subsequently confirmed by various studies as illustrated in **Figure 1**. Today, the commensal microbiota is being acknowledged as an integral part of the gut-brain axis and recognized to be an important modulator of the brain's physiology during all stages of life in health and disease. In addition, we know that the neuronal development is not only significantly impacted, but also orchestrated by the maternal microbiota (De Palma et al., 2015; Gomez de Agüero et al., 2016; Fleshner et al., 2017; Sarker and Peleg-Raibstein, 2018; Pasciuto et al., 2020; Vuong et al., 2020). The microbiota might act as an expected input to calibrate the development of the gut–brain axis and its absence or disruption during specific developmental windows is hypothesized to influence neuronal as well as neurocognitive development and ultimately behavior. Animal studies of social interactions provided strong evidence for microbial modulation of cognition and behavior. A robust relationship between social behavior and the microbiota was observed across species and led to the hypothesis that this relationship has an evolutionary basis (Cowan et al., 2020).

The influence of gut microbiota in neurodevelopment was recognized since the early 2000s by experiments using GF, antibiotic-treated, gnotobiotic and SPF mice. Researchers disrupted the microbiota of mice through antibiotic treatment which resulted in animals displaying several neurological problems (Sudo et al., 2004; Neufeld et al., 2011; Clarke et al., 2013; De Vadder et al., 2014; Daneman and Prat, 2015; Buffington et al., 2016) like impaired social behaviors (Cryan and Dinan, 2012; Buffington et al., 2016), reduced anxiety like behavior (Neufeld et al., 2011; Cryan and Dinan, 2012; Clarke et al., 2013; Cryan et al., 2019) and increased motor and rearing activity (Diaz Heijtz et al., 2011; Arentsen et al., 2015). The observed impairment of brain development and behavior could be ameliorated by reconstitution of newborn mice with a diverse and intact microbiome (Cryan and Dinan, 2012; O'Mahony et al., 2017; Cryan et al., 2019).

Swann and Heijtz have previously shown that the gut microbiota can influence brain physiology, for instance through the regulation of neurotransmission and synaptogenesis (Diaz Heijtz et al., 2011) as well as through the modulation of the metabolic profiles of the prefrontal cortex and hippocampus of rodents (Swann et al., 2017). In their newest study, they characterized the neurobiochemical profile of the forebrains of mice during three key postnatal developmental stages, co-occurring with the maturation of the gut microbiota. They could show that gut microbial-derived molecules are able to cross the blood–brain barrier (BBB) and that the gut microbiome may therefore influence neurodevelopmental trajectories. One example is *S*-adenosyl methionine, a compound which is most abundant in the neonatal forebrain and that acts as an essential methyl donor for DNA and histone methylation, a fundamental process for brain development and function (Swann et al., 2020).

It was shown that neurogenesis is influenced by microbiota since adult GF mice exhibit increased neurogenesis in the dorsal hippocampus compared to conventional laboratory mice (Ogbonnaya et al., 2015). This phenotype could not be reversed by colonization of GF mice at weaning and indicated that microbial signals very early in life reduce the rate of neurogenesis in the hippocampus. Further, GF mice differ in dendrite morphology and show increased volume of the amygdala and



Impact on	References	Title
Neuronal development	De Palma et al., 2015	Microbiota and host determinants of behavioral phenotype in maternally separated mice
	Gomez de Agüero et al., 2016	The maternal microbiota drives early postnatal innate immune development
	Fleshner et al., 2017	Danger signals and inflammasomes: Stress-evoked sterile inflammation in mood disorders
	Sarker and Peleg-Raibstein, 2018	Maternal overnutrition induces long-term cognitive deficits across several generations
	Pasciuto et al., 2020	Microglia require CD4 T cells to complete the fetal-to-adult transition
	Vuong et al., 2020	The maternal microbiome modulates fetal neurodevelopment in mice
	Swann et al., 2020	Developmental signatures of microbiota-derived metabolites in the mouse brain
Brain physiology	Diaz Heijtz et al., 2011	Normal gut microbiota modulates brain development and behavior
	Marín-Burgin and Schinder, 2012	Requirement of adult-born neurons for hippocampus-dependent learning.
	Ogbonnaya et al., 2015	Adult hippocampal neurogenesis is regulated by the microbiome
	Luczynski et al., 2016	Adult microbiota-deficient mice have distinct dendritic morphological changes: differential effects in the amygdala and hippocampus
	Möhle et al., 2016	Ly6C(hi) monocytes provide a link between antibiotic-induced changes in gut microbiota and adult hippocampal neurogenesis
	Swann et al., 2017	Application of 1 H NMR spectroscopy to the metabolic phenotyping of rodent brain extracts: A metabonomic study of gut microbial influence on host brain metabolism
	Fitzpatrick et al., 2020	Gut-educated IgA plasma cells defend the meningeal venous sinuses
Myelination	Keogh et al., 2021	Myelin as a regulator of development of the microbiota-gut-brain axis
BBB	Braniste et al., 2014	The gut microbiota influences blood-brain barrier permeability in mice
Microglia maturation	Erny et al., 2015	Host microbiota constantly control maturation and function of microglia in the CNS
	Quigley, 2017	Microbiota-brain-gut axis and neurodegenerative diseases
Aging	Golomb et al., 2020	Multi-modal single-cell analysis reveals brain immune landscape plasticity during aging and gut microbiota dysbiosis
	Li et al., 2020	Age-related shifts in gut microbiota contribute to cognitive decline in aged rats
	Madison and Kiecolt-Glaser, 2021	The gut microbiota and nervous system: Age-defined and age-defying

FIGURE 1 | The gut microbiota modulates the development, maturation, and aging of the CNS. The gut microbiota plays a pivotal role throughout all stages of life. It impacts brain physiology, myelination, blood–brain barrier function (BBB) as well as microglia maturation and contributes to the process of aging. Table lists corresponding references, all studies are primarily based on rodent data.

the hippocampus (Luczynski et al., 2016). Moreover, decreased neurogenesis in the hippocampus of adult mice was induced after long-term antibiotic treatment leading to deficits in the novel object recognition task. Treatment with probiotics and voluntary exercise were sufficient to rescue these phenotypes (Möhle et al., 2016).

Myelination of the peripheral and central nervous system occurs rapidly during early life and is critical in regulating motor, sensory as well as cognitive functions. Neonatal antibiotics (Abx)-induced dysbiosis dysregulates host-microbe interactions and myelination in the brain, thereby leading to decreased hippocampal neurogenesis, increased myelination in the pre-frontal cortex (PFC) and causes behavioral impairments of adult mice. The administration of the SCFA butyrate restored behavior and myelination impairments, suggesting a critical role of the gut microbiota in mediating these effects (Keogh et al., 2021).

In the last decade, there was a great number of results from studies using GF mice pointing to a key role of the commensal gut microbiota in early brain development and adult neurogenesis. These results confirmed – amongst others – the impact of microbiota on microglia maturation mediated via SCFAs and the modulation of astrocyte activity via tryptophan and aryl hydrocarbon receptors. The microbiota also influences

the activation of peripheral immune cells and the cytokine profile which affects – beside systemic and CNS inflammation – brain development (Quigley, 2017).

Recently it was shown, that the meninges, the membranes that surround the brain and spinal cord, contain IgA-secreting plasma cells that are educated in the gut. IgA traps microbes in the intestinal mucus to prevent a breach of the mucosal layer (Moor et al., 2017) and it was elucidated that they exert the same function at the dural venous sinuses, an important internal barrier interface. Seeding the meninges with antibody-producing cells recognizing gut commensals likely ensures a defense against the most common invaders originating from the gut, thereby preventing bacteremia in the CNS. Therefore, IgA cells emerging from the gut may act as an immunological firewall which is assembled during homeostasis to prevent spreading of pathogens into the meninges and the underlying CNS parenchyma during gut inflammation and breach of the intestinal barrier (Fitzpatrick et al., 2020).

In summary, current data clearly indicate that signals from the microbiota can regulate neurogenesis, apoptosis and synaptic pruning. Thus, the development of a healthy and functional brain depends on many pre- and post-natal events that integrate various environmental cues like molecular signals from the gut, largely originating from its microbiome that are communicated via the bidirectional gut-brain axis.

THE IMPACT OF THE GUT MICROBIOME ON THE HOMEOSTASIS AND AGING OF THE NERVOUS SYSTEM

In addition to a healthy neuronal development, there is growing evidence that the commensal gut microbiota also highly influences brain function and architecture under homeostatic conditions and during normal aging.

It was demonstrated that the BBB integrity in the frontal cortex, hippocampus and striatum is influenced by the commensal gut microbiota. Germ-free mice display an increased permeability of the BBB, this phenomenon is already evident during embryonal development due to reduced tight junctions by decreased expression of claudin 5 and occludin (Braniste et al., 2014). Recolonization of GF mice with complex commensal gut microbiota or the application of the SCFA butyrate restored the integrity of the BBB. Further, it was recently shown that the host microbiota controls microglia maturation and brain innate immune function. Adult GF mice show stunted microglia under homeostatic conditions compared to microglia from SPF mice (Erny et al., 2015). Microbial derived SCFAs are known to be able to cross the BBB (Huuskonen et al., 2004). The oral application of a mixture of the three SCFAs acetate, propionate and butyrate was sufficient to drive maturation of microglia, which suggests that they may affect microglia directly (Erny et al., 2015). Additionally, it was also shown that commensals have an effect on adult hippocampal neurogenesis leading to more immature neurons in GF mice but not in conventionally colonized mice (Marín-Burgin and Schinder, 2012; Ogbonnaya et al., 2015).

Normal aging is characterized by a progressive functional cognitive decline (Partridge, 2001) accompanied by significant changes in the gut microbiota of the elderly (Claesson et al., 2011). How aging and gut microbiota dysbiosis, which also occurs naturally while aging, reshapes brain immune cell plasticity and homeostasis is still not fully understood. Functional and phenotypical plasticity of brain immune cells contribute to brain tissue homeostasis as well as disease and is profoundly influenced by its microenvironment and systemic factors. Circulating metabolites and plasma cytokine composition are significantly altered by gut dysbiosis leading to dysregulation of the peripheral immune system (Arpaia et al., 2013; Bachem et al., 2019; Lehallier et al., 2019). CNS immunity and neuroinflammation is also indirectly altered by dysbiosis through gut microbiota-derived signaling molecules (Erny et al., 2015; Dinan and Cryan, 2017; Ma et al., 2019). There are emerging findings in animals and humans suggesting that the gut microbiota play a major role in gut-brain communication, ultimately shaping neurological aging trajectories by either helping to maintain nervous system function into late life or promoting pathology and positions the intestinal microbiota as an arbiter of age-related neurological decline (Madison and Kiecolt-Glaser, 2021). Gut microbiota dysbiosis can induce aberrant immune responses, which in turn disrupt the local and systemic homeostasis of the host and emerging evidence has highlighted the importance of gut microbiota in age-related diseases of the central nervous system such as AD and stroke. Several approaches like manipulating the microbiome via fecal microbiota transplantation (FMT), administration of prebiotics and probiotics as well as dietary interventions have been utilized to reduce age-related dysbiosis in experimental models and in clinical studies (Holmes et al., 2020).

Cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) was used to map brain immune cell plasticity in response to systemic perturbations of aging and gut dysbiosis to characterize the compositional and transcriptional plasticity of brain immunity. CITE-seq revealed transcriptional changes among inflammatory/patrolling Ly6C$^+$ monocytes and CNS-associated innate lymphoid cells (ILCs). The discovery of such immune cell plasticity during aging and gut dysbiosis can help to learn more about the critical components determining brain immunity in aging and to ascertain the onset of age-related neurodegenerative diseases (Golomb et al., 2020).

A recent study investigated the effect of aged gut microbiota on cognitive decline by using fecal microbiota transplantation from aged to young rats (Li et al., 2020). Their results exhibited that FMT impaired the cognitive behavior in the young recipient rats leading to decreased regional homogeneity in the medial prefrontal cortex and hippocampus, reduced expression of brain-derived neurotrophic factor (BDNF), N-methyl-D-aspartate receptor NR1 subunit and synaptophysin, changed synaptic structures and decreased dendritic spines and increased expression of advanced glycation end products (AGEs) and receptor for AGEs (RAGE). Additionally, following FMT young rats showed increased levels of oxidative stress and pro-inflammatory cytokines, indicating that oxidative stress and inflammation may underlie gut-related cognitive decline in aging.

Taken together, these studies point toward a critical involvement of gut microbiota in maintaining neuronal homeostasis as well as in normal and pathological aging. A well-balanced equilibrium between gut microbiota and the host appears to be essential in maintaining neuronal health while significant disturbances of this relationship can lead to a variety of psychiatric and neurological diseases, which we will discuss throughout the following paragraphs.

THE IMPACT OF THE GUT MICROBIOME ON DISORDERS OF THE CENTRAL NERVOUS SYSTEM

After discussing the impact of the microbiome on the healthy development, the homeostasis as well as normal aging of the nervous system we will now focus on how microbiota influence the two most common neurological diseases that likely impose the greatest socioeconomic burden on humanity in the field of neuroscience: Alzheimer's disease and stroke (**Figure 2**). In this context we will illustrate that our current mouse models were an excellent tool for proof-of-principal studies and therefore crucial in gaining fundamental insights into pivotal host-microbiota interactions. However, we will also discuss their weaknesses in the one-to-one translation of mouse model-based results into human clinical trials. Last but not least and based on the lessons learned throughout this review we propose a strategy that may increase the translational research value of current mouse models and that may help us to tap into the full therapeutic potential of translational microbiome research in the field of neuroscience.

Microbiota and Alzheimer's Disease

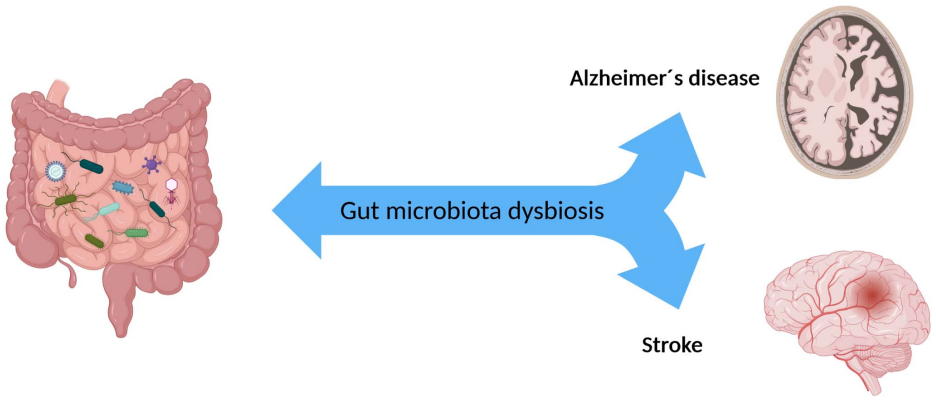
According to the World Health Organization AD is the most common form of dementia and by far the greatest factor causing disability and dependency among older people around the world (WHO, 2020). The clinical picture of AD has severe physical, psychological, social and economic effects, not only on people with AD, but also on their caregivers, families and the society as a whole; the socioeconomic consequences are dramatic and cannot be overstated. However, we still have no effective prevention and treatment strategy for AD (Zahs and Ashe, 2010; Cummings et al., 2014).

Alzheimer's disease is a degenerative disorder caused by polymerization of amyloid β leading to the progressive loss of neurons. Interestingly, it has been recognized that amyloid β can act as an antimicrobial peptide via activation of toll-like receptor 2 (Welling et al., 2015; Kumar et al., 2016). However, it possesses harmful properties in a dysregulated state. Gut microbiota are a source of amyloid proteins sharing some structural similarities with amyloids from the CNS. The immune system could be primed by bacterial proteins in the gut enhancing immune response to endogenous productions of neuronal amyloid in the brain or they may act via molecular mimicry as prion proteins eliciting cross-seeding (Kowalski and Mulak, 2019).

Chronic overexposure to the biometal manganese (Mn) leads to manganism and is reported to cause neurodegenerative disorders (Guilarte and Gonzales, 2015). Its neurotoxic ability can potentially induce alterations to neuropathological features like amyloid β and Tau aggregation, which induces neuroinflammation in an NLRP3 inflammasome dependent manner at the heart of AD and PD. It was recently shown, that Mn exposure exaggerates host amyloid β and Tau production in the brain and causes hippocampal degeneration and necrosis. Fecal microbiome transplantation from normal control rats into rats exposed to Mn resulted in reduced amyloid β and Tau expression and downregulated NLRP3 and other neuroinflammatory factors. Hence, remodeling the gut microbiota in Mn exposer could be used as a therapeutic strategy to dampen neuroinflammation (Wang et al., 2020).

It was shown, that the widely used APP/PS1 double transgenic mice that are expressing a chimeric mouse/human amyloid precursor protein (APP) and a mutant human presenilin 1 (PS1) had a remarkable shift in the gut microbiota diversity compared to non-transgenic wild-type mice. Additionally, germ-free APP/PS1 transgenic mice exhibit a drastic decrease in the level of cerebral amyloid β pathology versus healthy control mice with gut microbiota (Harach et al., 2017). Similar results in the shift of microbiota composition were observed in the transgenic APP/PS1 mouse model by Bäuerl and displays higher amounts of the inflammatory related *Erysipelotrichaceae* family (Bäuerl et al., 2018). Further, germ-free APP/PS1 mice were found to show a reduction in amyloid β pathology in contrast to conventional mice (Radde et al., 2006). Additionally, the $ADLP^{APT}$ mouse model expressing amyloid precursor protein, presenilin 1 and tau protein carrying human mutations, develops an AD-like pathology with amyloid and neurofibrillary tangles (Kim et al., 2018). They also show community level-alterations in the gut microbiota compared to wild-type mice. FMT from wild-type donor mice was able to alleviate the formation of amyloid β plaques and neurofibrillary tangles, glial reactivity and cognitive impairment in the recipient animals (Kim et al., 2020). In another common AD mouse model that expresses five familial AD mutations (5xFAD), it was demonstrated that the anti-inflammatory *Bifidobacterium longum*, which is able to suppress human gut microbiota LPS production and LPS-induced NF-κB activation in LPS-stimulated microglial BV-2 cells, from healthy human fecal microbiota could alleviate cognitive decline in 5xFAD transgenic and aged mice (Lee et al., 2019). In an effort to investigate the different effects of constitutive and induced microbiota modulation on microglia, 5xFAD mice bred under both GF and SPF conditions as well as mice with induced gut bacteria depletion by ABX were used (Mezö et al., 2020). The results revealed that constitutive or induced microbiota modulation differentially controls microglial amyloid β clearance mechanisms and prevents neurodegeneration and cognitive deficits. Actually, the constitutive absence of gut microbiota in GF 5xFAD mice enhanced the microglial uptake of amyloid β deposits in the hippocampus which led to a decreased amyloid β burden and associated neuronal loss as well as retained hippocampus-associated memory function.

Further, it was shown in a recent study that gut microbiota from AD patients can promote intestinal inflammatory response by activating intestinal NLRP3 inflammasome (Shen et al., 2020). Gut microbiota from AD patients were transplanted into



Impact on	References	Title
Aggravation of AD pathology	Harach et al., 2017	Reduction of Abeta amyloid pathology in APPPS1 transgenic mice in the absence of gut microbiota
	Kim et al., 2018	Molecular and functional signatures in a novel Alzheimer's disease mouse model assessed by quantitative proteomics
	Bäuerl et al., 2018	Shifts in gut microbiota composition in an APP/PSS1 transgenic mouse model of Alzheimer's disease during lifespan
	Wang et al., 2019	Sodium oligomannate therapeutically remodels gut microbiota and suppresses gut bacterial amino acids-shaped neuroinflammation to inhibit Alzheimer's disease progression
	Haran et al., 2019	Alzheimer's Disease Microbiome Is Associated with Dysregulation of the Anti-Inflammatory P-Glycoprotein Pathway
	Shen et al., 2020	New mechanism of neuroinflammation in Alzheimer's disease: The activation of NLRP3 inflammasome mediated by gut microbiota
	Kim et al., 2020	Transfer of a healthy microbiota reduces amyloid and tau pathology in an Alzheimer's disease animal model
	Mezö et al., 2020	Different effects of constitutive and induced microbiota modulation on microglia in a mouse model of Alzheimer's disease
	Wang et al., 2020	The gut microbiota attenuate neuroinflammation in manganese exposure by inhibiting cerebral NLRP3 inflammasome
Amelioration of AD pathology	Bourassa et al., 2016	Butyrate, neuroepigenetics and the gut microbiome: Can a high fiber diet improve brain health?
	Bonfili et al., 2017	Microbiota modulation counteracts Alzheimer's disease progression influencing neuronal proteolysis and gut hormones plasma levels
	Bonfili et al., 2018	SLAB51 probiotic formulation activates SIRT1 pathway promoting antioxidant and neuroprotective effects in an AD mouse model
	Rezaeiasl et al., 2019	The effects of probiotic *Lactobacillus* and *Bifidobacterium* Strains on memory and learning behavior, long-term potentiation (LTP), and some biochemical parameters in β-amyloid-induced rat's model of Alzheimer's disease
	Lee et al., 2019	Suppression of gut dysbiosis by Bifidobacterium longum alleviates cognitive decline in 5XFAD transgenic and aged mice
	Mehrabadi and Sadr, 2020	Assessment of probiotics mixture on memory function, inflammation markers, and oxidative stress in an Alzheimer's disease model of rats
Aggravation of stroke pathology	Singh et al., 2016	Microbiota dysbiosis controls the neuroinflammatory response after stroke
	Yamashiro et al., 2017	Gut dysbiosis is associated with metabolism and systemic inflammation in patients with ischemic stroke
	Stanley et al., 2018	An insight into intestinal mucosal microbiota disruption after stroke
	Ahnstedt et al., 2020	Sex differences in T cell immune responses, gut permeability and outcome after ischemic stroke in aged mice
	Jeon et al., 2020	Dynamic changes in the gut microbiome at the acute stage of ischemic stroke in a pig model
	Xu et al., 2021	Rapid gut dysbiosis induced by stroke exacerbates brain infarction in turn
Amelioration of stroke pathology	Wang et al., 2011	Valproic acid attenuates blood-brain barrier disruption in a rat model of transient focal cerebral ischemia: the roles of HDAC and MMP-9 inhibition
	Hasan et al., 2013	Effect of HDAC inhibitors on neuroprotection and neurite outgrowth in primary rat cortical neurons following ischemic insult
	Kim and Chuang, 2014	HDAC inhibitors mitigate ischemia-induced oligodendrocyte damage: potential roles of oligodendrogenesis, VEGF, and anti-inflammation
	Feng et al., 2017	Infiltration and persistence of lymphocytes during late-stage cerebral ischemia in middle cerebral artery occlusion and photothrombotic stroke models
	Jaworska et al., 2019	Effect of the HDAC inhibitor, sodium butyrate, on neurogenesis in a Rat model of neonatal hypoxia-ischemia: Potential mechanism of action
	Chen et al., 2019	Transplantation of fecal microbiota rich in short chain fatty acids and butyric acid treat cerebral ischemic stroke by regulating gut microbiota
	Feng et al., 2019	Chronic colitis induces meninges traffic of gut-derived T cells, unbalances M1 and M2 microglia/macrophage and increases ischemic brain injury in mice
	Jian et al., 2019	The involvement and therapy target of immune cells after ischemic stroke
	Liu et al., 2020	Communications between peripheral and the brain-resident immune system in neuronal regeneration after stroke
	Sadler et al., 2020	Short-chain fatty acids improve poststroke recovery via immunological mechanisms
	Lee et al., 2020	Gut microbiota-derived short-chain fatty acids promote poststroke recovery in aged mice

FIGURE 2 | Reciprocal effects of the gut microbiota on Alzheimer's disease and stroke. Numerous studies highlight that gut microbiota influences various CNS disorders such as Alzheimer's disease and stroke in a negative and/or positive fashion. CNS disorders can also act on gut microbiota composition via the gut-brain axis. Table lists corresponding references, all studies are primarily based on rodent data with the exception of reference (Yamashiro et al., 2017; Haran et al., 2019; and Jeon et al., 2020).

APP/PS1 double transgenic mice leading to increased expression of NLRP3 in the intestinal tract and increased expression levels of inflammatory factors in peripheral blood. In addition, activation of microglia in the central hippocampus of these mice as well as increased expression of neuroinflammatory factors was observed. Transplantation of healthy human gut microbiota was then used to improve the composition of gut microbiota leading to down-regulation of the intestinal expression of NLRP3, improved cognitive ability of the mice, suppressed activation of microglia in the central hippocampus and down-regulated expression of neuroinflammatory factors. Further, the intestinal expression of NLRP3 was upregulated after transplantation of gut microbiota from AD patients into C57BL/6 mice. Therefore, improving the composition of intestinal bacteria in AD patients can attenuate neuroinflammation caused by NLRP3.

Wang et al. (2019) investigated the dynamic changes of gut microbiota associated with neuroinflammation in AD with respect of microbial derived amino acid metabolites. They used 5xFAD and APP/PS1 mouse models and observed that alteration of gut microbiota composition leads to peripheral accumulation of phenylalanine and isoleucine, which are able to stimulate the differentiation and proliferation of pro-inflammatory T helper 1 cells (Th1). Once the peripheral Th1 cells have infiltrated the brain, they are associated with M1 microglia activation, which contributes to AD-associated neuroinflammation. Additionally, they used a clinical phase 3 sodium oligomannate drug that was shown to improve cognition to target the gut microbiota. This drug could suppress gut dysbiosis together with the associated phenylalanine/isoleucine accumulation, restrained neuroinflammation and reverses cognition impairment.

There is growing evidence that probiotics and prebiotics beneficially modulate microbial and immune pathways to improve neurological disorders and brain functions (Suganya and Koo, 2020). AD rats treated with probiotics for 4 weeks exhibit significantly improved spatial learning and memory, long-term potentiation, paired-pulse facilitation ratios and lipid profiles (Rezaeiasl et al., 2019) along with decreased amyloid β plaques and reduced oxidative and inflammatory markers within 10 weeks (Mehrabadi and Sadr, 2020). Another probiotic formulation used in AD mice attenuated cognitive impairment, brain injuries, amyloid β aggregation and alternation of neuronal proteolysis (Bonfili et al., 2017) plus antioxidant and neuroprotective effects via activation of the SIRT1 pathway (Bonfili et al., 2018). Additionally, it has been reported that prebiotics may improve brain function and prevents neurological disorders like AD (Kinney et al., 2018).

The SCFA butyrate is synthesized in the colon by the commensal gut microbiota via fermentation of otherwise non-digestible fiber (Pryde et al., 2002) and can improve brain health. Butyrate uses diverse modes of action to execute its beneficial effect on various brain disorders: (i) as energy metabolite to produce ATP playing a role in metabolism and mitochondria activity (e.g., in AD, PD, stroke, mitochondrial encephalopathy, and adrenoleukodystrophy), and (ii) its epigenetic ability to serve as a histone deacetylase inhibitor (e.g., AD, PD, and stroke). This wide array of biological functions makes butyrate to a very attractive therapeutic molecule to prevent neurodegeneration and to promote regeneration (Bourassa et al., 2016). Interestingly, elderly patients with AD carry a lower abundance of butyrate producing bacteria and instead a higher proportion of taxa that are associated with neurological disorders and taxa that are known to cause proinflammatory states. Furthermore, it was demonstrated that stool samples from elderly AD patients induced lower production of anti-inflammatory p-glycoprotein *in vitro* than samples from elders without AD or with other dementia types (Haran et al., 2019).

Around 50 million people worldwide suffer from dementia and there are almost 10 million new cases reported every year (WHO, 2020). Although AD is with 60–70% by far the most common cause of progressive dementia, there is still neither a prophylactic nor an effective therapeutic strategy against this devastating disease (Scheltens et al., 2016). The WHO estimates the current proportion of dementia cases in the population over the age of 60 years at approximately 5–8%. Importantly, the total number of dementia patients - according to current estimates - is expected to reach 82 million in 2030 and up to 152 million in 2050 (WHO, 2020), imposing a significant global health and socioeconomic burden on humanity that will, if it progresses uncontrolled, overwhelm our healthcare systems. This dramatic situation demands – from an economical, ethical and social perspective – the highest possible prioritization of research into AD and the fastest possible development of new and effective treatments. Microbiome research may have the inherent potential of providing answers, at the very least to some of these challenges. Most basic research on AD has predominantly used wildtype and genetically modified mice (GF, antibiotic-treated, gnotobiotic, and SPF) to recapitulate the genetic and pathological elements of human AD, thereby deriving mechanistic and therapeutic knowledge that is hoped to translate to the human condition. The translational goal of basic research for medical diseases in all fields is to provide the knowledge required to predict, prevent, identify, treat, and hopefully cure these diseases in human patients. As such, the success of this approach for AD is rather poor. For instance, as reviewed by Zahs and Ashe, over 200 different interventions have been reported to be effective in the APP mouse model of AD, but none has proven effective in human trials (Zahs and Ashe, 2010). In fact, the rate of AD drugs entering human trials and fail was 96.4% from 2002 to 2012 (Cummings et al., 2014) leading to the emerging challenge of producing animal based results that better translate to human outcomes.

As already described by scientists in 1989, Alzheimer's patients exhibit plaque deposits not only in the brain, but also in the intestine (Joachim et al., 1989), indicating a linkage of both organs in the context of AD. With age, the integrity of the BBB and the gastrointestinal epithelium decline (Tran and Greenwood-Van Meerveld, 2013) as well as the composition of the gut microbiome (Woodmansey, 2007) changes with age, and it is known that age is the greatest risk factor for AD. In addition, many genes associated with AD are expressed in the gut and several microbial factors have been linked to AD pathogenesis (Brandscheid et al., 2017). This indicates that we need mouse models with gut microbiota and immune systems that will allow scientists to better translate the findings of gut microbiota-AD interaction into the human system.

Microbiota and Stroke

According to the WHO, the cerebrovascular accident also known as stroke is the second leading cause of death and the third leading cause of disability worldwide (WHO, 2012). There are over 13.7 million new cases of strokes reported each year (GBD 2016 Stroke Collaborators, 2019) and globally, one in four people over the age of 25 will have a stroke in their lifetime (Feigin et al., 2018). Since the gut microbiome is known to significantly influence vascular diseases, its impact on the cerebrovascular disease stroke appears obvious and logical as reviewed elsewhere (Tang et al., 2017; Durgan et al., 2019; Battaglini et al., 2020; Verhaar et al., 2020). Moreover, this connection comes with the inherent potential of developing microbiota-related therapeutics.

In the context of stroke, the delicate interplay of the bidirectional communication between the gut microbiota and the CNS along the gut-brain-axis is also widely discussed leading to the question whether gut dysbiosis is the cause or the consequence of stroke. Post stroke, the commensal microbiota change in favor of opportunistic pathogens, which may be due to the systemic release of cytokines and chemokines produced in the brain, changed gut motility and permeability as well as mucus production leading to dysbiosis. Dysbiosis subsequently worsens the outcome of stroke. However, gut dysbiosis has also been associated to reinforce risk factors for acute ischemic stroke like hypertension, diabetes, obesity, vascular dysfunction or the changed microbiota during aging (Battaglini et al., 2020).

Several animal-based studies have demonstrated substantial differences in microbiota composition even at the phylum level between poststroke models and controls in all sections of the gastrointestinal tract like increased abundance of *Akkermansia muciniphila* and clostridial species (Stanley et al., 2018). Changes of gut microbiota were already visible within 3 days after stroke (Singh et al., 2016) and included a reduced diversity of microbiota species, intestinal bacterial overgrowth with a preferential expansion of the Bacteroidetes phylum, and more specific stroke-induced changes on the bacterial genus and at the species level (Yamashiro et al., 2017).

Since the elderly experience profound systemic responses after stroke resulting in more severe long-term disability and higher mortality, a recent study investigated if restoring youthful gut microbiota after stroke benefits recovery in aged subjects (Lee et al., 2020). Gut microbiota from young mice were gavaged into aged mice 3 days after induction of ischemic stroke resulting in less behavioral impairment as well as reduced brain and gut inflammation. Microbial sequencing and metabolomics analysis revealed that young fecal transplants contained much higher SCFA levels and four related bacterial strains were used for a more defined transplantation. The four selected SCFA-producers were able to attenuate post-stroke neurological deficits and inflammation in aged stroke mice. In addition, they were able to boost concentrations of SCFA in the gut, the plasma as well as the brain. These results suggest that the poor stroke recovery in aged mice can be reversed via post-stroke bacteriotherapy of youthful gut microbiota.

Observation of a clinical cohort of patients with ischemic stroke revealed a fast induction of intestinal ischemia and an excessive production of nitrate resulting in gut dysbiosis with expansion of *Enterobacteriaceae* (Xu et al., 2021). Overgrowth of *Enterobacteriaceae* magnifies brain infarction by enhancing systemic inflammation and is a potential risk biomarker for a poor primary outcome of patients. A mouse model with middle cerebral artery occlusion also showed fast gut dysbiosis based on stroke-induced intestinal ischemia and reperfusion leading to nitrate respiration causing enrichment of *Enterobacteriaceae*. The increase of *Enterobacteriaceae* accelerates systemic inflammation through the LPS-TLR4 pathway and aggravated brain infarction. The overgrowth of *Enterobacteriaceae* could be suppressed by blocking nitrate generation or nitrate respiration which reduced the systemic inflammation and further ameliorates brain infarction.

Interestingly, stroke displays some well-known sex differences. Recent data adjusted for age and pre-stroke functional status revealed that while women account for more stroke deaths, men display a higher overall mortality. A current study investigated in aged mice the sex differences in immune response, gut permeability and microbial diversity after stroke induction. In males, stroke induced greater gut permeability and non-reversible alterations in microbiota diversity along with greater neurological deficits and impaired sensorimotor function as well as greater cognitive decline (Ahnstedt et al., 2020).

A recent study utilizing a middle cerebral artery (MCA) occlusion ischemic stroke pig model evaluated the changes in gut microbiota composition and diversity past stroke (Jeon et al., 2020). After ischemic stroke induction, blood and fecal samples were taken at different time points and revealed ameliorated systemic inflammation with elevated plasma levels of TNF-α and IL-6 12 h after the stroke. The microbial diversity was already reduced at day 1 post stroke and almost normalized by day 5 indicating dynamic changes and plasticity of the gut microbiome in an acute period of stroke.

As shown for AD, intestinal microbiota can also benefit stroke outcome. Gut microbiota can promote neuronal regeneration via immune-related signaling and metabolites that can directly alter the phenotypes of resident immune cells. Circulating immune cells that have been shaped by immune components including the gut microbiota and their metabolites can infiltrate the brain and influence neuronal regeneration directly or through modulation of the properties of brain-resident immune cells in an ischemic brain (Liu et al., 2020). Thereby, gut microbiota and associated metabolites could be a component of a repair system after stroke that can strengthen repair-promoting immune responses or weaken neurotoxic immune responses to promote neuroplasticity (Feng et al., 2017; Jian et al., 2019).

Since it was shown that lymphocytes migrate from the gut to the brain after stroke, the altered gut microbial composition after stroke may shape the local immune environment in the brain in favor of neurogenesis and axon growth. It was observed that gut-derived $CD4^+$ T cells migrate after an ischemic injury to the meninges and control the balance between M1 and M2 microglia/macrophage (Feng et al., 2019). SCFAs can activate and recruit T cells to the brain, which then regulate the function of brain-resident microglia to promote synaptic plasticity after stroke in male mice (Sadler et al., 2020). Additionally, SCFAs can stimulate phenotype transition of microglia and exert

neurogenesis and neurite outgrowth permissive effects under ischemic conditions (Hasan et al., 2013; Jaworska et al., 2019). Butyric acid supplementation or SCFA-enriched fecal microbiota transplantation have both been shown to promote neurological recovery in adult neurogenesis after stroke (Chen et al., 2019). Further, sodium butyrate is able to reduce the infarct size in ischemic stroke models along with limited brain damage and improved behavioral outcomes (Kim et al., 2007; Langley et al., 2008; Wang et al., 2011; Kim and Chuang, 2014).

Taken together, gut microbiota is also a hot topic in the field of stroke research and was already critically discussed in the early days of stroke-microbiota research. Back then, researchers raised awareness for the fact that the gut contains not just bacteria but also viruses and bacteriophages, that most research is performed in GF mice and that we have to consider that they may deviate from normal physiology and have underdeveloped immune structures that might deceive results. Indeed, stroke research is highly struggling with a poor clinical translation rate and discussed in depth elsewhere (Pound and Rebecca, 2020). The vast majority of interventions for stroke successfully tested in preclinical animal studies have turned out to either have no efficacy (Horn and Limburg, 2001; Shuaib et al., 2007) or to be harmful to humans (Saxena et al., 1999; Bath et al., 2000; Davis et al., 2000; Enlimomab Acute Stroke Trial Investigators, 2001) and increased the risk of death in clinical trials. From more than 1,000 candidate neuroprotective drugs that where tested in animals, not a single one was found to benefit humans with stroke (O'Collins et al., 2006) and animal studies were just used to establish dosing but did not play a direct role in clinical translation (Pound and Rebecca, 2020). Additionally, just 2 out of approximately 500 compounds that have been reported to effectively reducing the effects of acute ischemic stroke in animal models have proven to be effective in humans. Besides drug development, behavioral studies continue the translational disappointments: While voluntary physical activity improves long-term stroke outcome in mice (Gertz et al., 2006), a higher rate of serious adverse events was observed in the aerobic group of subacute stroke patients compared with a relaxation group that received relaxation sessions (Nave et al., 2019). Thus, also the field of stroke urgently needs animal models that better recapitulate the human system for a more successful transfer from interventions from basic research and preclinical animal studies to human clinical trials.

CONCLUDING REMARKS

Finally, we want to propose future strategies that may help to tap into the full therapeutic potential of translational microbiome research in the field of neuroscience.

Particularly, the mammalian gut microbiome produces a recently unappreciated, but remarkably diverse set of biologically active products such as small molecule metabolites and their precursors. These compounds do not only exert local effects within the gut, they are also capable of breaching the intestinal barrier subsequently affecting various gut-distal sites such as the nervous system as described throughout this review. It is likely that these molecules play a dominant role in orchestrating the communication along the gut-brain axis (Diaz Heijtz et al., 2011; Collins et al., 2012; Wu et al., 2012; Forsythe et al., 2014; Sampson and Mazmanian, 2015; Yano et al., 2015; Kipnis, 2018). Hence, translational microbiome research, especially in the field of neuroscience, should aim to identify these compounds and to understand their biological function in health and disease. This approach may open up a promising window of opportunity and may be a pathbreaking way to discover novel drugs for the treatment of a broad variety of human diseases (Brown and Hazen, 2017).

In this context, it is pivotal to emphasize that the gut microbiota is an extremely complex and diverse ecosystem comprising microorganisms of all kingdoms – not just bacteria – that communicate with their host and with each other through biologically active compounds in a multifactorial and non-linear way (Norman et al., 2014; Pfeiffer and Virgin, 2016; Stappenbeck and Virgin, 2016). This was illustrated by several recent studies highlighting the impact of non-bacterial organisms of the microbiome on host physiology such as protists (Chudnovskiy et al., 2016), parasites (Howitt et al., 2016), fungi (Ackerman and Underhill, 2017), and viruses (Lim et al., 2016). Consequently, experimental models of translational microbiome research that are aiming to decipher mechanisms should benefit from a more complete description of the microbiome. Nevertheless, most studies still keep their focus exclusively on the bacterial microbiome, merely one component of the entire microbiome, which makes it not only difficult to decipher complex microbiota-related mechanisms, but also inherently restricts our chances of identifying novel, biologically active microbial compounds. Thus, a full description of the microbiome and a more comprehensive view of mammalian organisms as holobionts might be a key for the development of novel microbiota-based therapeutics.

Germ-free, antibiotic-treated, gnotobiotic, and SPF mouse models were essential to unravel basic principles as regards to the importance of gut microbiota in development, maturation, aging, homeostasis as well as function of the brain in health and disease. These lessons learned were fundamental in illuminating the therapeutic potential lying within microbiome research such as the use of microbial bio-active metabolites in the treatment of neurological diseases. However, the overwhelming amount of rodent-based data could not be directly translated from bench to bedside practice (Fisher et al., 1996; Mestas and Hughes, 2004; von Herrath and Nepom, 2005; Suntharalingam et al., 2006; Payne and Crooks, 2007; Seok et al., 2013; Shay et al., 2013; Garner, 2014; Hay et al., 2014; Mak et al., 2014; Wong et al., 2019; Pound and Rebecca, 2020) as we have demonstrated for AD (Zahs and Ashe, 2010; Cummings et al., 2014) and stroke (Saxena et al., 1999; Bath et al., 2000; Davis et al., 2000; Enlimomab Acute Stroke Trial Investigators, 2001; Horn and Limburg, 2001; Gertz et al., 2006; O'Collins et al., 2006; Shuaib et al., 2007; Nave et al., 2019; Pound and Rebecca, 2020). This raises the question of how the scientific community could optimize translational research models to better reflect human physiology in health and diseases. Given the vast impact of the microbiome on the immune and nervous system, it appears likely that the field of microbiome research might help to

address these issues. In this context, recent paradigm-shifting work illustrated that conventional laboratory mice are too far removed from natural environmental conditions to faithfully mirror the physiology of free-living mammals like humans. These mice lack the diverse microbial exposure that humans experience from birth and throughout their whole life, which is integral for immune maturation as well as immune experience and consequently the orchestration of immune responses. When working with conventional laboratory mice, this ultimately leads to false assumptions of how the human immune system works as reviewed elsewhere (Masopust et al., 2017; Graham, 2021).

In an effort to optimize mouse models of translational research, several approaches such as cohousing of conventional laboratory mice with pet store mice (Beura et al., 2016), sequential infections (Reese et al., 2016), rewilding in semi-natural habitats (Leung et al., 2018), engraftment of wild mouse gut microbiota into pregnant germ-free mice (Rosshart et al., 2017) and the transfers of conventional laboratory mouse embryos into wild mouse surrogate mothers (Rosshart et al., 2019) – the so-called wildling model – were proposed. Particularly wildlings accurately phenocopied the human outcome and could have prevented catastrophically failed clinical trials (Rosshart et al., 2019), where conventional laboratory mice as well as rat and non-human primate models had failed to predict the human response to harmful drug treatments (Fisher et al., 1996; Suntharalingam et al., 2006). Moreover, when compared to conventional mice, such animals were protected in models of various infectious diseases as well as cancer (Beura et al., 2016; Rosshart et al., 2017), which may allow for the identification of microbiota-mediated protective mechanisms that cannot be discovered in conventional laboratory mice. The idea of naturalizing mouse models for immunology to improve their translational potential was just recently discussed (Masopust et al., 2017; Graham, 2021).

Even though and likely due to the very recent appearance of these new mouse models, there is only limited direct evidence (Cope et al., 2019) that the use of such models will also improve the translational research value of mouse models in the field of neurosciences. Nevertheless, since we know that the microbiome and the immune system play an important role in mammalian physiology and particularly in health and disease of the CNS, it seems likely that the described models are a promising tool to also enhance the translational research value of mouse models in the context of neuroscience. Hence, adding these novel models to our experimental toolbox in neuroscience may facilitate the discovery of disease mechanisms and treatments that cannot be found in GF, antibiotic-treated, gnotobiotic and conventional SPF mice, increase the safety and success rate of bench-to-bedside efforts, reduce costs and ultimately accelerate the development of new disease treatments.

In conclusion, based on the profound knowledge scientists gained as regards to the impact of microbiota on the nervous system in health and diseases, we believe in the importance and therapeutic potential of microbiome research conducted in the context of neuroscience. However, to access the full therapeutic potential of microbiome research and subsequent drug discovery and development, we propose to (i) foster the holistic view on mammals as holobionts and to study all members of the microbiome as well as their biologically active products and metabolites and (ii) to take advantage of newly developed translational research models that more closely resemble the human meta-organism. This approach may help to unleash the full potential of microbiome research regarding the discovery of novel microbiota-related therapeutics of numerous neurological disorders of global relevance.

AUTHOR CONTRIBUTIONS

MS wrote the manuscript. MS and SR designed the figures. SR supervised and edited the manuscript. Both authors listed and approved it for publication.

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frontiers in Microbiology

REVIEW
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Colonizing Microbes, IL-10 and IL-22: Keeping the Peace at the Mucosal Surface

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Our world is filled with microbes. Each multicellular organism has developed ways to interact with this microbial environment. Microbes do not always pose a threat; they can contribute to many processes that benefit the host. Upon colonization both host and microbes adapt resulting in dynamic ecosystems in different host niches. Regulatory processes develop within the host to prevent overt inflammation to beneficial microbes, yet keeping the possibility to respond when pathogens attempt to adhere and invade tissues. This review will focus on microbial colonization and the early (innate) host immune response, with special emphasis on the microbiota-modifying roles of IL-10 and IL-22 in the intestine. IL-10 knock out mice show an altered microbial composition, and spontaneously develop enterocolitis over time. IL-22 knock out mice, although not developing enterocolitis spontaneously, also have an altered microbial composition and increase of epithelial-adherent bacteria, mainly caused by a decrease in mucin and anti-microbial peptide production. Recently interesting links have been found between the IL-10 and IL-22 pathways. While IL-22 can function as a regulatory cytokine at the mucosal surface, it also has inflammatory roles depending on the context. For example, lack of IL-22 in the IL-10–/– mice model prevents spontaneous colitis development. Additionally, the reduced microbial diversity observed in IL-10–/– mice was also reversed in IL-10/IL-22 double mutant mice (Gunasekera et al., 2020). Since in early life, host immunity develops in parallel and in interaction with colonizing microbes, there is a need for future studies that focus on the effect of the timing of colonization in relation to the developmental phase of the host. To illustrate this, examples from zebrafish research will be compared with studies performed in mammals. Since zebrafish develop from eggs and are directly exposed to the outside microbial world, timing of the development of host immunity and subsequent control of microbial composition, is different from mammals that develop *in utero* and only get exposed after birth. Likewise, colonization studies using adult germfree mice might yield different results from those using neonatal germfree mice. Lastly, special emphasis will be given to the need for host genotype and environmental (co-housing) control of experiments.

Keywords: IL-10, IL-22, zebrafish, microbiota, mice, epithelial homeostasis, intestines

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MICROBIAL COLONIZATION

As soon as organisms come into contact with the outside world the process of colonization starts. In some animals colonization starts at birth, while in others (such as fish) colonization of the eggs immediately starts when the eggs of the female are released into the surrounding water (Egerton et al., 2018). At 3 days post fertilization (dpf) the mouth of the fish opens and microbes can colonize the developing gastrointestinal tract (Kimmel et al., 1995). From the microbial perspective, colonization of a niche is often driven by the availability of substrates that allows growth and population expansion. As with any microbial colonization process of a new niche, it is initiated by pioneers, which are followed or sometimes replaced by other groups of organisms. The microbial ecosystem of host-associated niches commonly becomes more complex during these secondary stages, which are often including the colonization by species that can utilize the metabolic products of the pioneers as substrate for growth, thereby, establishing syntrophic chains and assembling an interactive microbial food-web (Sung et al., 2017; D'Hoe et al., 2018). Competition and cooperative processes will simultaneously lead to a more or less stable microbial community which we call microbiota.

Colonization of the gastrointestinal tract depends on several biotic (nutrients, other microbes, etc.) and abiotic (pH, temperature, etc.) factors. Furthermore, the onset of colonization can be different for different species. For example, colonization in mammals begins at birth (although there is some discussion on the sterility of the womb, Perez-Munoz et al., 2017), while in fish, spawned eggs directly acquire a microbiota (Liu et al., 2014). Therefore, the selective pressures on and later within the host, but also the (timing of the) development of immune responses toward microbes can be very different depending on the host species studied.

Recently, a large cohort study (TEDDY), typing 903 children (12,500 stool samples) from six different locations (European and United States), showed that during early life in humans, three distinct phases of microbial community structure could be discriminated: a developmental phase in roughly the first year, a transitional phase in the second year (<30 months), and a stable phase from ≥31 months (Stewart et al., 2018). Breastfeeding explained most microbial composition variance in the developmental phase, with those children receiving breastmilk showing a modestly higher abundance of *Bifidobacterium* (but also *Lactobacillus rhamnosus* and *Staphylococcus epidermidis*) compared to children that were formula fed that showed (modest) higher abundance of *Escherichia coli*. The metabolic genes enriched in the microbiome of breastfed children correlated with the presence of high concentrations on human milk oligosaccharides (HMOs) in breastmilk. The change into the transition phase seemed to be caused by the cessation of breastmilk rather than the start of introduction of solid food, confirming previous studies (Bergstrom et al., 2014; Backhed et al., 2015; Pannaraj et al., 2017). Infant formula is frequently supplemented with galacto- and/or fructo-oligosaccharides, which (in part) probably compensates for the difference in Bifidobacterial abundance during the earlier stages of life, explaining why smaller scaled studies may not have detected the association of this genus with breastfeeding (Timmerman et al., 2017).

The distinct phases of microbial community shifts were also reported in zebrafish. Longitudinal evaluation of the microbial composition in siblings from a single parent pair that were housed in separate tanks revealed that the microbiota followed a distinct developmental pattern (Stephens et al., 2016). In the early time points [4–10 days post fertilization (dpf)] the larval zebrafish microbiota was more similar to the environmental samples, and the diversity of the population was relatively high. Around 10 dpf this began to shift, resulting in two larval microbial subpopulations, one resembling the richness of the 4 dpf samples, the other group shifting more toward older samples. At this time point of 10 dpf, zebrafish should have begun feeding. This is a pivotal life-stage in the larvae, since failure to feed from 5 dpf onward results in starvation and death between 10 and 12 dpf (Samuel et al., 2019). It is likely that the ability to feed causes the bimodal distribution in the 10 dpf samples. Over time in the zebrafish the relative abundance of Proteobacteria decreases (although it remains the dominant phylum) and the relative abundance of Fusobacteria and Firmicutes increases (Rawls et al., 2004, 2006; Roeselers et al., 2011; Stephens et al., 2016). Furthermore, the richness and phylogenetic diversity decreases from larval to adult stage (Stephens et al., 2016). This microbiota data was also used in a microbial modeling study which revealed that especially during early developmental stages, passive migration and stochastic demographic processes play an important role in microbial succession and colonization (Burns et al., 2016).

Rearing zebrafish under different environmental conditions until 12 days post hatching (12 dph, ∼14 dpf) and subsequently changing the environment and sampling until 98 dph showed that ecological succession of gut microbial communities mainly associates with developmental stages rather than the hatching environment (Xiao et al., 2021). Additional research is required to decipher the influence of neutral versus non-neutral processes (selective pressures) that might influence different microbes within the microbial composition at different stages of life.

For the colonizing microbes, the intestine is a specific niche where they find food, but also experience selective pressures. Here, oxygen level, feed, and the developing immune system play important intertwined roles (Bevins and Salzman, 2011). In a meta-analysis, Sullam et al. (2012) investigated the gut microbiota of different fish species with different habitats and feeding behavior. Although differences exist between different environments, fish species and trophic levels, the fish gut microbiota still clustered with gut communities from other species (mammals and insects) and not with free-living environmental bacteria, showing that the gut environment is a remarkably consistent selective ecological niche (Sullam et al., 2012). Microbes also adapt to living within this specific niche. For example, *Bacteroides* has been shown to adapt by upregulating so-called commensal colonization factors (ccf) when in contact with the mouse colon, but not under laboratory culture conditions (Lee et al., 2013). In another experiment, *Aeromonas veronii* was used as a model species and 'passaged' through populations

of germfree larval zebrafish (Robinson et al., 2018). Each time the gut-associated *Aeromonas* population was inoculated in the aquatic environment of the next zebrafish population. Here, the authors showed that early adaptations in the microbe seemed to enhance initial colonization, while later adaptations were involved in host specialization (Robinson et al., 2018).

HOST–MICROBE INTERACTIONS

Next to adaptation by the colonizing microbes, the host also responds to these new colonizers. The host needs to launch adequate defense reactions to counteract microbes that may cause damage to its mucosal surfaces, but at the same time it needs to be permissive to those bacteria that do not cause harm, or even support the host's nutrient digestion processes. The host response can be well monitored by the exposure of germfree animals to conventional animal housing, enabling a new microbiota to be established. This process is also termed conventionalization. A large part of the host genes induced by conventionalization are immune-related and metabolic genes (El Aidy et al., 2012, 2013a,c; Hooper et al., 2012; Belkaid and Naik, 2013). Comparing conventionalization of zebrafish and mice showed that this response toward the microbiota is partly conserved between different host species (Rawls et al., 2004). Colonizing 3 dpf germfree zebrafish with bacteria from conventional zebrafish resulted in a transcriptional response of 212 genes (Rawls et al., 2004). Remarkably, but perhaps not surprisingly given the largely conserved pattern recognition receptors reacting to microbes in zebrafish and mammals (Li et al., 2017), 59 of these microbial colonization response genes were also modulated in mice upon microbial colonization. These genes were predominantly representing pathways involved proliferation, metabolism and (innate) immune responses, underpinning the conservation of the microbial colonization response across host species.

El Aidy et al. (2012, 2014) conventionalized adult germfree C57BL/6J mice and followed the host response as well as the microbial community over time. Interestingly, within one day after conventionalization, the bacterial log copy number reached it maximal size, not increasing further over the course of the experiment, indicating that a full-sized microbiota can establish quickly. In the first two days, the diversity remained low, but reached maximum levels around day 8 after conventionalization. Interestingly, at day 4 post conventionalization several known pathobionts (*Helicobacter, Sphingomonas,* and *Mucispirillum*) increase rapidly in abundance, coinciding with the highest activity of the innate immune system (measured by mucosal pro-inflammatory responses and plasma cytokine/amine levels). Subsequently, a sharp decrease in these pathobionts was observed between 8 and 16 days, coinciding with the increase of (innate and T cell-associated) immune activation and regulation (El Aidy et al., 2014). It can be hypothesized that microbial activation of innate immunity early in development creates a niche for species that can best resist this inflammatory environment (pathobionts), and subsequently their bloom induces adaptive immunity that in turn controls these species and restores the balance, thereby affecting microbial community structure.

Moreover, microbes can manipulate the host to gain a competitive advantage within the gut microbiome community. For example, *B. thetaiotaomicron* was shown to induce fucosylation of host surface glycans, which it can use as a substrate for growth (Bry et al., 1996; Hooper et al., 1999). Another example is given by Round et al. (2011), where it was shown that *Bacteroides fragilis* acts via the toll-like receptor (TLR)2 on T helper cells to ensure microbial symbiosis. Deletion of TLR2 specifically on T helper cells lead to activation of an anti-microbial response that limits *B. fragilis* colonization (for review of more host–microbe interactions by which microbes establish competitive advantages within the host's gut microbiota, see Round et al., 2011; Stevens et al., 2021). Likewise, some pathogenic species have been shown to benefit from the host immune response eliminating their competitors. One illustrative example was given in a competition experiment using mice, *Haemophilus influenzae* colonization of the nasopharynx induced recruitment of host neutrophils and stimulated killing of complement-opsonized *Streptococcus pneumoniae*. This increase in neutrophils was not seen when mice were colonized by *Haemophilus* alone (Lysenko et al., 2005). This last study, like other similar studies showing interhost competition, also illustrates the caveat of studying cell–cell interactions looking at one or a few selected species, compared to investigating an entire microbial community of several hundred species (Scales et al., 2016; Wang et al., 2020; Kern et al., 2021). The net results of all these interacting bacteria, the environment and the host, might not be adequately modeled by studying interactions of each individual species in isolation.

Not only does microbial colonization of the intestines induce metabolic and immunological changes, it is well-established that this colonization is even necessary for the development of homeostatic metabolic and immune processes in the host. A prerequisite to be able to mount immune responses is the timing of colonization in relation to the development of the host. This is different when for example comparing mammals to fish. In mammals both innate and adaptive immune cells are present in the periphery at birth, while in fish only innate immune cells are present from 2 dpf (at time of hatching). The first adaptive cells (T cells) leave the thymus from 10 dpf onward. Adaptive immunity in fish is thought to be fully mature around 3–4 weeks post fertilization (wpf) when antibody responses can be measured, although more research is currently ongoing on the exact timing in different fish species (Lam et al., 2004; Brugman, 2016; Dee et al., 2016). Of interest is to understand the timing of immune maturation in relation to the colonizing microbes (e.g., different cells will be present at different life stages of the host). The zebrafish is especially suited to understand how innate and adaptive immune processes orchestrate the microbial composition in the intestine. Especially, the sequential development of innate and adaptive immunity develop enables investigation of the influence of early innate responses in the absence of peripheral (intestinal) adaptive immunity until 10–14 days post fertilization.

Taken together, the microbial composition in the gut is dynamic, especially during early life. The dynamic interactions between microbes and the host over time stimulates adaptation

(gene regulation) and possibly evolution (loss or gain of functions) of the microbes (and eventually also the host, albeit at slower pace). During the lifetime of the host, regulatory processes ensure mucosal homeostasis. Regulatory cytokines IL-10 and IL-22, the focus of this review, are important factors in the maintenance of mucosal homeostasis between microbes and the host in the intestine. Aberrant signaling of these cytokines is often seen in (human) inflammatory disorders of the gut, such as inflammatory bowel disease (IBD) (Engelhardt and Grimbacher, 2014; Keir et al., 2020). For example, individuals with a missense mutation in either their IL-10 gene or its receptor genes my develop very early-onset IBD (Glocker et al., 2009; Kotlarz et al., 2012; Shouval et al., 2014). Next to IL-10, it is well known that polymorphisms in the IL-23R gene are associated with IBD (Neurath, 2019). As IL-23 signaling is also required for IL-22 induction, these polymorphisms might also implicate disturbances of IL-22 signaling in IBD susceptibility. Since IL-22 is both involved in regulatory as well as inflammatory processes, its precise role during IBD onset, flares and remission are currently unknown, but have been reported to be altered in IBD patients (Schmechel et al., 2008). In the next sections, the role of IL-10 and IL-22 in microbiota control is discussed.

FUNCTION OF IL-10 IN CONTROLLING THE GUT MICROBIAL COMMUNITY

IL-10 is produced by a variety of immune cells, of which major IL-10 producers include dendritic cells, macrophages, innate lymphoid cells (ILC2s) and regulatory T cells, and to a lesser extent by neutrophils, B cells, other T lymphocyte-subsets and even epithelial cells (Izcue et al., 2006; Saraiva and O'Garra, 2010; Ueda et al., 2010; Rivollier et al., 2012; Olszak et al., 2014; Seehus et al., 2017; Mishima et al., 2019; Miyamoto et al., 2019; Bando et al., 2020). IL-10 exerts its action by binding to the heterodimeric IL-10 receptor, consisting of IL-10R1, expressed on immune and other hematopoietic cells, and IL-10R2, which is widely expressed and can also bind to other cytokines such as IL-22 (co-receptor IL-22R1). Binding of IL-10 to its receptor activates Jak1 and Tyk2 and subsequently leads to phosphorylation of STAT3. In turn, STAT3 induces Socs3 expression, that inhibits pro-inflammatory cytokine pathways (Donnelly et al., 1999; Riley et al., 1999; Fillatreau and O'Garra, 2014). In this way IL-10 suppresses chemokine expression and downregulates co-stimulatory molecules [CD80, major histocompatibility complex (MHC) II] (Kasama et al., 1994; Willems et al., 1994; Wei et al., 2020). Furthermore, IL-10 decreases pro-inflammatory cytokine expression by inhibiting transcription factor NF-kB (Driessler et al., 2004). IL-10 also promotes the induction and maintenance of regulatory T cells (Tregs). Specifically, Tregs are induced when antigen presentation by dendritic cells occurs concomitantly with an instructive IL-10/TGF-β milieu (Izcue and Powrie, 2008). In addition, IL-10 maintains Foxp3 expression (a master regulator in the development and function of Tregs) in these cells during inflammation. These Tregs may further suppress inflammatory responses (Izcue et al., 2006; Murai et al., 2009). IL-10–/– mice develop spontaneous colitis (Kühn et al., 1993). However, neither germfree IL-10–/– mice nor their derivatives that are mono-associated with certain pathobionts (e.g., *H. hepaticus*) develop this disease, suggesting that colonization with a complex microbiota may be a prerequisite for disease development in these mice (Sellon et al., 1998; Dieleman et al., 2000). Intriguingly, various studies suggested that differences in the composition of such complex microbiota strongly affects disease severity (Kim et al., 2005; Sydora et al., 2005; Büchler et al., 2012; Yang et al., 2013).

Several studies reported differences in gut microbiota composition between mice that lack IL-10 and their wildtype counterparts (summarized in **Table 1**). Multiple factors may confound data on microbiota composition, indicating the importance of controlling for these factors as much as possible. Firstly, given the importance of environmental stimuli in shaping the microbiota, it is important to co-house experimental animals to eliminate cage- or tank-specific effects. Secondly, to minimize the effect of different genotypes (ensuring the genetic variation is only in the gene of interest) the use of littermates would be ideal (for a comprehensive review, see Laukens et al., 2016). Differences in gut microbiota composition were demonstrated by conventionalization of adult germfree IL-10–/– and WT littermates that led to distinct microbiota development in these animals (Maharshak et al., 2013). While the microbiota diversity and richness decreased over time in the IL-10–/– mice, this was not seen in WT littermates. In contrast, WT littermates displayed an increasing diversity until the final timepoint analyzed at 2 weeks post conventionalization (Maharshak et al., 2013). Specifically, the abundance of Proteobacteria (*E. coli*) increased over time in the conventionalized IL-10–/– mice, which coincided with the activation of spontaneous inflammation and the onset of colitis. Analogously, increased *E. coli* abundance was also observed in IL-10–/– mice that were conventionally raised at a timepoint that colonic inflammation was also apparent (at 8–10 weeks of age) in these animals (Maharshak et al., 2013). Unfortunately, the microbiota composition of these conventionally raised animals was not investigated at earlier ages when inflammation was still absent, disallowing the analysis of the time-dependent co-development of the microbiota and the inflammatory responses during early life stages. Such studies could reveal microbiota changes that occur prior to the onset of spontaneous colitis, while the present study observed differences in microbial composition between wildtype and IL-10–/– littermates may very well be a consequence of the inflammatory milieu that selects for bacteria that can resist such conditions, like *E. coli* (Thiennimitr et al., 2011). Moreover, in the latter experiments the IL-10–/– mice and WT littermates were not co-housed, and the differences detected may partially depend on cage effects. Several studies confirm the elevated *E. coli* abundance in IL-10–/– mice (Arthur et al., 2012, 2014; Overstreet et al., 2021). Unfortunately, these studies also do not include samples taken prior to inflammation onset, and do not compare between co-housed IL-10–/– and wildtype littermates, which disables the exclusion of several environmental factors that can confound the microbiota comparisons. However, prolongation of the analyses of the IL-10–/– mice associated

TABLE 1 | Summary of studies investigating the role of lack of IL-10 on the development of colitis and microbiota composition.

	Animal model	Intervention	Analysis	Timeline	Littermates/ Co-housed	Findings	References
IL-10$^{-/-}$	Mouse	Conventionally and SPF raised mice	Development of colitis	Analysis at 0–12 weeks of age	Littermates, not co-housed	IL-10$^{-/-}$ mice develop spontaneous colitis	Kühn et al., 1993
	Mouse	GF, SPF and colitis-related-bacteria-colonized mice	Development of colitis	GF: 0–6 months SPF: 0–38 weeks Colitis-related-bacteria-colonized: adults; analysis 5–32 weeks post-colonization	Littermate status not clear, not co-housed	Development of spontaneous colitis in IL-10$^{-/-}$ mice is dependent on the presence of microbiota	Sellon et al., 1998
	Mouse	Mono-colonization with *Helicobacter hepaticus*	Development of colitis	2-month old mice; analysis 7–16 weeks post-colonization	Littermates; not co-housed	*H. hepaticus* mono-colonization did not induce colitis in IL-10$^{-/-}$ mice	Dieleman et al., 2000
	Mouse	SPF; in different facilities; infection with *H. hepaticus*	Microbiota composition and susceptibility to colitis severity	Analysis at 38, 141, 172, and 204 days of age	Not littermates; not co-housed	Susceptibility to *H. hepaticus*-induced colitis differed among facilities, related to differing microbiota compositions	Yang et al., 2013
	Mouse	Mono-colonization with *H. hepaticus* and SPF infected with *H. hepaticus*	Microbiota composition and susceptibility to colitis severity	8-week old mice; analysis 4 and 13 weeks post-colonization	Not co-housed	Changes in gut microbiota composition are involved in strain-specific susceptibility of IL-10$^{-/-}$ mice to colitis	Büchler et al., 2012
	Mouse	Mono-colonization with specific bacterial species	Susceptibility to colitis severity	Analysis 7 and 22 weeks post-colonization	Not littermates; not co-housed	Bacterial mono-colonization did not induce colitis in IL-10$^{-/-}$ mice, despite stimulating a systemic response	Sydora et al., 2005
	Mouse	Mono-colonization with commensal bacterial species	Susceptibility to colitis severity	10–12 weeks old mice; analysis 1–46 weeks post-colonization	Not littermates; not co-housed	Different commensal bacteria initiate different patterns of intestinal inflammation	Kim et al., 2005
	Mouse	Embryonic transfer into dams differing in microbiota composition; infection with *H. hepaticus*	Microbiota composition and disease severity	Infection at 24–26 days of age; analysis at 111 days of age;	Littermates, co-housed and maternal microbes tested	Disease severity can be influenced by microbiota composition, which depends on maternally inherited gut microbiota and host genotype	Hart et al., 2017
	Mouse	(1) GF IL-10$^{-/-}$ and WT colonized with same donor microbiota; (2) SPF raised IL-10$^{-/-}$ and WT	Microbiota composition	(1) Adults; analysis 1–4 weeks post-colonization (2) Analysis at 8 and 10 weeks	Littermates; not co-housed	Over time, WT mice had increased gut microbiota diversity and richness, IL-10$^{-/-}$ mice had decreased diversity and richness, with increase in Proteobacteria (*Escherichia coli*). SPF raised mice showed similar increase in *E. coli*	Maharshak et al., 2013

(Continued)

TABLE 1 | (Continued)

	Animal model	Intervention	Analysis	Timeline	Littermates/ Co-housed	Findings	References
	Mouse	SPF IL-10$^{-/-}$ and WT	Microbiota composition	Analysis at 4–19 weeks of age	Not littermates; not co-housed	Microbiota diversity was similar between young WT and IL-10$^{-/-}$ mice, but IL-10$^{-/-}$ mice did not show increased microbial complexity as WT did, together with an increase in *E. coli* and a reduction in *Clostridium* in IL-10$^{-/-}$ mice	Overstreet et al., 2021
	Mouse	GF IL-10$^{-/-}$ and WT colonized with SPF microbiota	Microbiota composition	Analysis at 2–20 weeks post-colonization	Not littermates; not co-housed	Increase in *E. coli* in IL-10$^{-/-}$ mice at 2 weeks; *E. coli* abundance decreased over time in IL-10$^{-/-}$ and WT, but remaining higher in IL-10$^{-/-}$	Arthur et al., 2012, Arthur et al., 2014
	Mouse	(1) SPF IL-10$^{-/-}$ and WT (2) SPF IL-10$^{-/-}$ and WT treated with antibiotics	Paneth cell function	(1) analysis at 7 weeks old (2) 9 weeks old mice; analysis at 11 weeks	Not littermates; not co-housed	More immature granules in Paneth cells of IL-10$^{-/-}$ mice. Without microbiota, Paneth cells were more mature, but with more amorphous granules	Berkowitz et al., 2019
	Mouse	IL-10$^{-/-}$ and WT	Bacterial penetration/ localization	Analysis at 8–12 weeks of age	Not littermates, not co-housed	More penetrable mucus layer and direct bacteria-epithelium contact in IL-10$^{-/-}$ mice	Johansson et al., 2014
	Zebra-fish	IL-10$^{-/-}$ and WT	Development of colitis	Analysis at 1 year old	Siblings; not co-housed	IL-10$^{-/-}$ zebrafish do not show any signs of intestinal inflammation at baseline	Harjula et al., 2018
	Mouse	IL-10–/–IL-22–/– and IL-10–/–	Development of colitis and microbiota composition	16–20 weeks old mice	Not littermates; not co-housed	IL-22 deficiency prevents spontaneous colitis in IL-10–/– mice Both IL10–/– and IL-10–/–IL-22–/– display more Th17 cells. Microbial diversity lower in IL-10–/– but same as wildtype in IL-10–/–IL-22–/– mice	Gunasekera et al., 2020
IL10R–/–	Mouse	SPF IL-10Rb(IL-10R2)$^{-/-}$ and IL-10Rb(IL-10R2)$^{\pm}$	Microbiota composition	Analysis at 0–30 weeks of age	Littermates; co-housed	Inflammation is dependent on microbiota in IL-10Rb$^{-/-}$ mice; No difference in microbiota composition and diversity between IL-10Rb$^{-/-}$ and IL-10Rb$^{\pm}$	Redhu et al., 2017

GF, germfree; IL, interleukin; SPF, specific pathogen free; WT, wildtype.

microbiota to later ages established that in both the wildtype and IL-10–/– mice the abundance of *E. coli* went down over time, albeit that at each timepoint the *E. coli* abundance was higher in the IL-10–/– mice compared to their wildtype counterpart (Arthur et al., 2012, 2014). Taken together these findings suggest that the initial colonization in WT mice with higher levels of *E. coli*, followed by microbiota succession toward a more Firmicutes and Bacteroides dominated microbiota, is also occurring in IL-10–/– animals although at a slower rate. None of these studies is able to decide whether the observed higher abundance of *E. coli* plays a causative role in the increasing inflammation in the IL-10–/– mice or is the consequence of the inflammatory milieu. The delayed microbiota maturation toward a Bacteroidetes and Firmicutes dominated ecosystem in the IL-10–/– mice, could suggest that the IL-10–/– mice might have difficulty regulating/controlling Enterobacterial species. Their initial colonization is likely to trigger a state of 'transient' inflammation in the wildtype mice that is balanced by regulatory responses that establish appropriate homeostatic relations with typical intestinal microbial phyla of the Bacteroidetes and Firmicutes, while suppressing enterobacterial species, such as *E. coli* (Renz et al., 2011; Al Nabhani et al., 2019). This secondary regulatory response is absent in IL-10–/– mice, which may lead to intestinal inflammation directly and may eventually drive toward colitis. Intriguingly, a recent report showed that IL-10–/– mice contain more immature granules in their Paneth cells, suggesting a role for IL-10 in Paneth cell function modulation (Berkowitz et al., 2019). Especially since Paneth cells are the predominant producers of anti-microbial peptides that play an important role in keeping the bacteria away from the replicating stem cell niche. Disturbed secretion of antimicrobial factors might explain (part of) the observed loss of microbial containment in the IL-10–/– intestine. In addition, it was also shown that although the inner mucus layer in the colon seemed to be even thicker in IL-10–/– mice, it was shown to be more penetrable by bacteria compared to that of wildtype mice (Johansson et al., 2014).

Interestingly, Fung et al. (2016) showed that a population of 'lymphoid resident bacteria' (specific members of the α-proteobacteria and β-proteobacteria) exist that are able to colonize and survive in murine dendritic cells. Dendritic cells colonized with members of these lymphoid resident bacteria expressed higher levels of IL-10 (and IL-1β and IL-23) compared to uncolonized dendritic cells. In the absence of IL-10, an almost 10-fold increase in Th17 + cells in the mesenteric lymph nodes and Peyer's patches was observed, which might indicate that IL-10 induced in the lymphoid tissues by these lymphoid tissue resident commensals (inside dendritic cells) is able to directly limit Th17 expansion and create a tolerogenic environment in the lymphoid tissues (Fung et al., 2016).

Arguing against a role for IL-10 signaling in shaping the microbial composition is the observation that IL-10 receptor b–/– (IL-10rb–/–) mice did not differ in their microbial composition and diversity from their littermates (IL-10rb+/–) (Redhu et al., 2017). Yet, the authors do describe that a few significant minor operational taxonomic units (OTU) differences were observed at different ages of the animals, however, these were not further specified. An important difference with the other studies described above is that the mice used in this study were littermates that were co-housed in a specific pathogen free facility.

In an effort to unravel the link between host genotype, environmental factors and severity of colitis in IL-10–/– mice, Hart et al. (2017) performed an embryonic transfer study. IL-10–/– embryos were implanted in surrogate dams which were allowed to naturally deliver and raise the pups. IL-10–/– pups born from surrogate mothers with different backgrounds developed a different microbiota and displayed different disease susceptibility when subsequently colonized with *H. hepaticus*. In this study IL-10–/– embryos were taken from different background [C57BL/6 (B6) and C3H/HeJBir (C3H)] and surrogate dams could be harboring the Charles River, Taconic farms or Jackson farms microbiota. As has been shown previously, animals derived from these different farms harbor a different microbiota. This has consequences for the presence (percentage) of different immune cell subsets in the intestines (Ivanov et al., 2009). Interestingly, in the B6 IL-10–/– mice increased microbial diversity was found in those pups born from mothers harboring the Charles River microbiota and these pups also had the lowest disease score after colonization with *H. hepaticus* compared to the B6 IL-10–/– mice raised by mothers harboring Taconic or Jackson farm microbiota. On the C3H background, IL-10–/– mice born to surrogate dams from Jackson or Taconic farms had increased disease severity (but also increased microbial diversity) compared to those born from surrogate dams harboring Charles River microbes. For both C3H and B6 IL-10–/– mice, those raised by Charles River containing surrogate dams had the lowest relative abundance of *H. hepaticus*. So this study illustrates that disease severity and microbial composition (including the ability of the disease causing *H. hepaticus* to colonize) depends on the gut microbiota of the surrogate dams as well as on the genetic background of the pups (Hart et al., 2017). This last study really emphasizes the need to use littermates and to co-house the animals studied, especially when looking at dynamic and interactive processes such as colonization and immune development.

In conclusion, the impact of IL-10 on the composition of the microbiota is far from clear and properly controlled experiments are needed. Sampling should be performed over time, including early time points that precede the onset of colitis, and employing littermates (IL-10+/+ and IL-10–/–) that are co-housed. One could expect an effect of IL-10 on microbial composition, if only based on the fact that IL-10 plays a role in the maintenance and development of IgA + plasma cells that are reported to have an impact on the microbial composition (Kunisawa et al., 2013; Huus et al., 2021). This might indicate that timing of sampling is crucial, since developing intestinal inflammation in these IL-10–/– mice might obscure more subtle dysbiotic processes early on. To investigate early innate regulation in the absence of adaptive immunity, the zebrafish model might be useful, as zebrafish develop from eggs *ex utero* and solely rely on innate immunity in the first 1–2 weeks of life (Brugman, 2016; Dee et al., 2016). Notably, mutant zebrafish lacking functional IL-10 did not develop spontaneous colitis, but did express increased levels of interferon (IFN)-γ in the gills (Harjula et al., 2018; Bottiglione et al., 2020). Moreover, inflammatory responses in

the gills upon exposure to irritants (Resiquimod R848) were stronger in IL-10 deficient zebrafish compared to wildtypes, although this difference could only be shown in later phases of the inflammatory response (Bottiglione et al., 2020). Since colitis in IL-10–/– mice is clearly associated with presence of certain members of the microbiota, further investigation into the microbial composition and possible disease inducing potential of (specific or combinations of) microbes in IL-10–/– zebrafish could shed some light on loss or gain of IL-10 and the effect on the microbial composition in health and disease.

Next to microbial composition analysis, determinants of host barrier function (Paneth cell function, epithelial proliferation and mucus structure) should be investigated in parallel, especially at early time points that precede full-blown inflammation, in order unravel the multiple factors contributing to dysbiosis and loss of homeostasis in IL-10–/– animals. An important factor in epithelial barrier function regulation is IL-22. In the next section the role of IL22 in keeping the peace at the mucosal surface is addressed.

FUNCTION OF INTERLEUKIN 22 IN GUT MICROBIAL COMPOSITION

IL-22 belongs to the IL-10 family of cytokines, together with IL-19, IL-20, IL-24 and in humans, IL-26 (Parks et al., 2015; Shohan et al., 2020). Production of IL-22 occurs particularly at mucosal surfaces. While many cells like macrophages, neutrophils, $CD4^+$ Th17, Th22, γδ T cells, LTi cells, and natural killer T cells have all been shown to produce IL-22, group 3 ILCs are probably its major innate cellular source (Liang et al., 2006; Cella et al., 2009; Colonna, 2009; Eyerich et al., 2009; Ouyang et al., 2011; Rutz and Ouyang, 2011; Hansson et al., 2013; Zindl et al., 2013; Yeste et al., 2014; Dudakov et al., 2015).

IL-22 exerts its actions via binding to the IL-22 receptor, which consists of two subunits: IL-22R1 and IL-10R2 (Dumoutier et al., 2000; Xie et al., 2000; Kotenko et al., 2001a,b; Li et al., 2004). While IL-22 is mainly produced by immune cells, it is targeting non-immune cells; in the intestines IL-22R is expressed mainly on epithelial cells (Shohan et al., 2020). In general, IL-22 promotes antimicrobial activity by stimulating antimicrobial peptide production by epithelial cells, inducing mucus production and supporting tissue repair (Radaeva et al., 2004; Sugimoto et al., 2008; Zheng et al., 2008). Furthermore, IL-22 may also promote intestinal stem cell (ISC) expansion (Lindemans et al., 2015).

IL-22 has been reported to have both regulatory and inflammatory effects depending on the context. Low level IL-22 production and signaling (during homeostasis) seems to strengthen epithelial barrier function, while increased levels of IL-22 induced during inflammation help clearing bacterial infections, such as shown for *Citrobacter rodentium* infection (Munoz et al., 2015; Tsai et al., 2017). Secreted IL-22 receptor, IL-22 binding protein (IL-22BP) (produced by dendritic cells) is upregulated during inflammation and might be a regulator of overt inflammatory effects (reviewed in Parks et al., 2015). In the next section, we will focus on the microbiota shaping potential of IL-22. An overview of studies making use of IL-22–/– animals is given in **Table 2**.

An important role for keeping the peace via IL-22 might be via innate lymphoid cell (ILC) function. For example, in the absence of ILCs, *Alcaligenes* species could disseminate (and cause systemic disease) in mice, which could be prevented by the administration of IL-22 (Sonnenberg et al., 2012). Furthermore, IL-22 has been shown to induce the proliferation of epithelial cells, strengthening the intestinal barrier (Ouyang et al., 2011; Lindemans et al., 2015). Recently, it was shown that by using Paneth cell-specific IL-22Rα (IL22R1)–/– mice, that Paneth cell maturation and antimicrobial effector function are dependent on IL-22R signaling (Gaudino et al., 2021). C57BL/6 Paneth cell specific IL-22R–/– ($IL\text{-}22Ra1^{fl/fl}$;Defa6-cre) mice displayed reduced expression of Paneth cell-specific Lyz1, Mmp7, and some α -defensins.

Since IL-22 induces antimicrobial peptides and mucins it is likely that IL-22 has intestinal health and microbiota modulating effects (Wolk et al., 2004; Liang et al., 2006; Wolk et al., 2006; Boniface et al., 2007; Sugimoto et al., 2008; Zheng et al., 2008). Indeed, it was shown that α-IL-22 antibody administration to germfree mice colonized with a human microbiota changed the composition of the gut microbiota (Nagao-Kitamoto et al., 2020). Further research showed that IL-22 modulated glycosylation of host N-linked glycans in this model. In this humanized microbiota mouse model, these host glycans promoted the growth of succinate consuming bacteria which sequestered the succinate from use by *C. difficile*, preventing outgrowth of *C. difficile* and disease in the intestine (Nagao-Kitamoto et al., 2020). This microbiota modulating effect by IL-22 via effects on glycosylation was also shown in IL22R–/– mice. Although no differences in microbiota composition were observed between IL22R–/– mice and wildtype mice at baseline, it was shown that IL-22R signaling protected against lethal *Citrobacter rodentium infection* by increasing colonization resistance to other disease-enhancing pathobionts such as *E. faecalis* (Pham et al., 2014). It was shown that IL22R signaling promotes the expression of Fut2, required for epithelial cell surface fucosylation. This fucosylation has been shown to shape the intestinal microbiota by favoring colonization of some (beneficial) species, while preventing others (pathobionts) like *E. faecalis* to colonize (colonization resistance) (Marcobal et al., 2011). Administration of fucosylated oligosaccharides to *C. rodentium*-challenged IL-22R–/– mice decreased the colonization of *E. faecalis* and attenuated *C. rodentium* infection (Pham et al., 2014). Also, administration of fucosylated oligosaccarides increased the diversity and composition of the microbiota in IL-22R–/– mice during *C. rodentium* infection. Therefore, it seems that IL22 signaling is able to restore the diversity of anaerobic commensal symbionts by increasing *Fut2*-mediated fucosylation on epithelial cells thereby favoring colonization of beneficial bacteria.

The aforementioned Paneth cell specific IL-22R–/– mice, that show decreased anti-microbial peptide production, also showed an altered microbial composition (Gaudino et al., 2021). 16S DNA sequencing of co-housed littermates revealed increased colonization of α-Proteobacteria and Peptostreptococcaceae in the terminal ileum of Paneth cell specific IL-22R–/– mice.

TABLE 2 | Summary of studies investigating the role of lack of IL-22 on the microbiota composition.

	Animal model	Intervention	Analysis	Timeline	Littermates/ Co-housed	Findings	References
IL-22$^{-/-}$	Mouse	SPF IL-22$^{-/-}$ and WT	Microbiota composition and susceptibility to colitis severity	Start DSS treatment: 8–12 weeks of age; start co-housing: 4–6 weeks pre-DSS treatment; analysis 1 week post-DSS initiation	Not littermates and littermates; separately and co-housed	IL-22–/– more susceptible to colitis induction. Transfer of IL-22–/– microbes to WT in co-housing. IL-22 seems to shape balance between immunity and colonic microbiota	Zenewicz et al., 2013
	Mouse	Ltbr$^{-/-}$ (lack IL-23 and IL-22) and WT	Microbiota composition	Analysis at 13 weeks of age	Littermates	Lack of IL-22 and IL-23 was associated with an increase in small intestinal epithelial-attaching SFB	Upadhyay et al., 2012
	Mouse	SPF Ahr$^{-/-}$ (less IL-22 production)	Microbiota composition	Analysis at 6–10 weeks of age	Littermates	Ahr$^{-/-}$ mice show increased epithelial-attaching SFB	Qiu et al., 2013
	Mouse	IL-22$^{-/-}$, Rag$^{-/-}$/IL-22$^{-/-}$ and WT; inoculation with *Achromobacter* + antibiotics treatment	Bacterial lymphoid tissue colonization	6–12 weeks of age; analysis 10 days post inoculation	Co-housed or littermates	Lack of IL-22 prevented *Achromobacter* colonization of lymphoid tissue	Fung et al., 2016
	Zebra-fish	Morpholino knock-down of IL-22; exposure to *Aeromonas hydrophila* or LPS	Susceptibility to bacterial infection	*A. hydrophila* infection at 2 dpf; analysis at 3 dpf	Siblings, not co-housed	Knock-down of IL-22 increased mortality in absence of infection; exposure to *A. hydrophila* or LPS increased pro-inflammatory cytokine expression and reduced survival with IL-22 knock-down	Costa et al., 2013
	Mouse	SPF Rag$^{-/-}$ (no B and T cells) mice with ILC depletion/anti-IL-22 mAb treatment; administration of IL-22	Microbial translocation	Adult	n.a.	Lack of ILCs led to bacterial translocation of lymphoid tissue resident *Alcaligenes* and systemic inflammation, which could be prevented by IL-22 administration	Sonnenberg et al., 2012

(Continued)

TABLE 2 | (Continued)

	Animal model	Intervention	Analysis	Timeline	Littermates/ Co-housed	Findings	References
	Mouse	SPF atherosclerosis-prone IL-23$^{-/-}$, IL-22$^{-/-}$ (via bone marrow reconstitution); fed WD	Microbiota composition	Start experiments: 6 week old; analysis 8–16 weeks post-WD feeding	Littermates; co-housed	Inactivation of IL-23-IL-22 signaling reduced intestinal barrier function and increased epithelial adherent bacteria and increased systemic LPS	Fatkhullina et al., 2018
	Mouse	IL22-antibody treatment	Microbiota composition during *C. difficile* infection	Rag1–/–, WT, 8- to 16-week-old female and male mice	No littermates, not co-housed	IL-22 modulated glycosylation promoted the growth of succinate consuming bacteria which sequestered the succinate from use by *C. difficile*, preventing outgrowth of *C. difficile* and disease in the intestine	Nagao-Kitamoto et al., 2020
	Mouse	IL-10–/–IL-22–/– and IL-10–/–	Development of colitis and microbiota composition	16–20 weeks old mice	Not littermates; not co-housed	IL-22 deficiency prevents spontaneous colitis in IL-10–/– mice Both IL10–/– and IL-10–/–IL-22–/– display more Th17 cells. Microbial diversity lower in IL-10–/– but same as wildtype in IL-10–/–IL-22–/– mice	Gunasekera et al., 2020
IL22R–/–	Mouse	IL22R1–/–	Antimicrobial effector functions during *C. rodentium* infection/DSS and microbial composition	Adult mice DSS 7 days *C. rodentium* 10–25 days post infection	No littermates, not co-housed	IL-22R signaling protected against lethal *Citrobacter rodentium* infection by increasing colonization resistance to other disease-enhancing pathobionts such as *E. faecalis*	Pham et al., 2014
	Mouse	SPF paneth cell specific IL-22R1$^{-/-}$	Antimicrobial effector functions and microbial composition	Analysis at 6–8 weeks of age	Littermates; co-housed	Paneth cell antimicrobial effector function depends on IL-22Ra1 signaling. Paneth cell specific IL-22R deletion was associated with an altered microbial composition (increased SFB)	Gaudino et al., 2021

Ahr, aryl hydrocarbon receptor; dpf, days post fertilization; DSS, dextran sodium sulfate; IL, interleukin; ILC, innate lymhoid cells; LPS, lipopolysaccharide; Ltbr, lymphotoxin beta receptor; mAb, monocloncal antibody; SFB, segmented filamentous bacteria; SPF, specific pathogen free; WD, western diet; WT, wildtype.

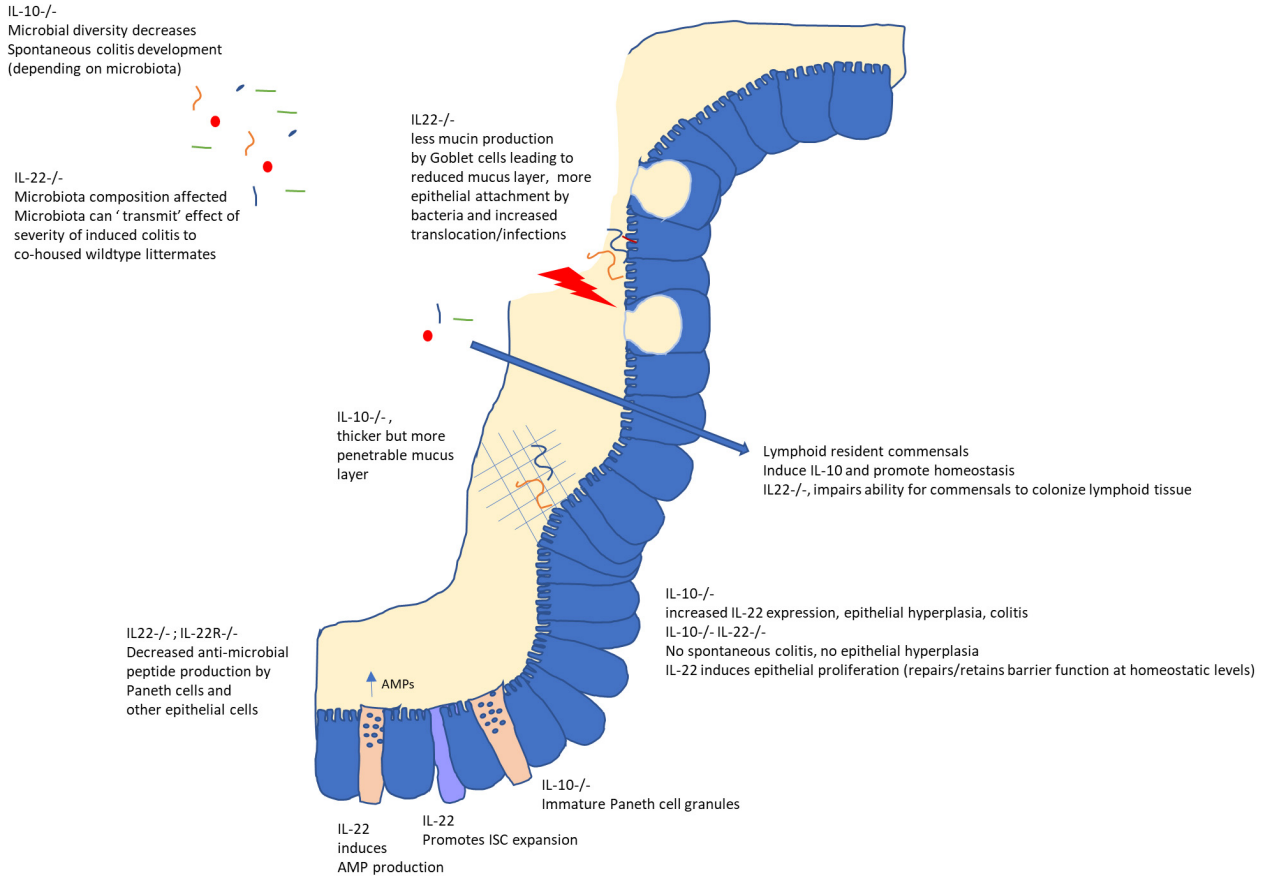

FIGURE 1 | Role of IL-10 and IL-22 on the intestinal mucosal barrier and microbiota. While IL-10 is important to maintain homeostasis in the gut, IL-22 plays a dual role. In the absence of inflammation or infection, IL-22 signaling is important for epithelial proliferation, anti-microbial peptide (AMP) secretion and mucus secretion by Goblet cells as well as possibly allowing (IL-10-inducing) colonization of lymphoid tissue by selected microbes. However, in the case of infection or inflammation, increased IL-22 augments inflammatory processes as evidenced by its role in colitis in IL-10–/– mice and its requirement to, for example, combat *C. rodentium* infections. Interestingly, microbiota from IL22–/– mice could confer increased severity of chemically induced colitis to wildtype littermates, indicating these effects on the host (AMPs, mucin secretion) might also lead to an altered microbial composition.

Quantitative PCR showed an increase in epithelial cell attaching segmented filamentous bacteria in the ileum of these mice. The fact that in the absence of IL-22, the host is unable to prevent epithelial attachment has been shown consistently. For example, mice lacking the lymphotoxin β receptor (which have decreased IL23 and IL-22 production) and Ahr–/– mice (in which ILC3s produce less IL-22), all displayed an increased levels of epithelial-attaching segmented filamentous bacteria (Upadhyay et al., 2012; Qiu et al., 2013). Likewise, low density lipoprotein receptor (ldlr) knock out mice (model for atherosclerosis) with a deletion of either IL23 or IL-22 in the bone-marrow derived cells (immune cells) exhibited reduced intestinal barrier function and increased epithelial adherent bacteria including Clostridiaceae and Ruminococcaceae, causing systemic increase in lipopolysaccharide (LPS) and trimethylamine *N*-oxide (TMAO) associated with development of atherosclerosis (Fatkhullina et al., 2018). On the other hand, pathogens are sometimes able to use the induction of antimicrobial protein expression induced by IL-22 to their advantage. It has been shown that antimicrobial proteins, such as lipocalin-2 and calprotectin, can sequester essential metal ions from microbes, thereby inhibiting their growth (Behnsen et al., 2014). However, some pathogens that can overcome this metal ion starvation can benefit and make use of this competitive advantage (Behnsen et al., 2014). IL-22-induction of antimicrobial peptides therefore does not automatically lead to homeostasis, but is influenced by the continuous evolutionary rat race between host and pathogens.

While several studies show different mechanisms by with IL-22 might modulate certain species within the microbiota, Zenewicz et al. (2013) investigated whether IL22–/– mice displayed differences in the total gut microbiota by also controlling for host genetic background and environmental effects, something that most studies neglect. While IL-22–/– mice did not show different colonic architecture compared to wildtype mice, they do develop more severe colitis in response to dextran-sodium sulfate (DSS) treatment compared to wildtype mice (Zenewicz et al., 2013). Interestingly, cohousing wildtype

mice with IL-22–deficient mice increased the severity of DSS-induced colitis in these wildtype mice, suggesting that the colitis might be 'transmissible' as has been shown for TRUC mice (mice that are deficient for both T-bet and Rag2) (Garrett et al., 2007). This suggests that transmissible agents, most likely (members of) the microbiota can 'transmit' this increased severity. The IL-22–/– mice still developed more severe colitis upon DSS treatment than the wildtype mice when co-housed, and therefore both the lack of IL-22 function in the host, as well as the ('transmissible') microbiota of these mice play a role in the severity of DSS-induced colitis. To tease apart the effects of host genotype and housing, heterozygous siblings (IL-22+/–) were in crossed to obtain a colony of wildtype, heterozygous and mutant IL-22 mice, in which the mice were siblings and co-housed and colitis was induced by DSS (Zenewicz et al., 2013). In this sibling and co-housing controlled experiment, again the wildtype mice co-housed with the IL-22–/– showed increased wasting disease compared to wildtype not co-housed with their IL-22–/– siblings (Zenewicz et al., 2013). Fecal samples were taken of these mice prior to DSS treatment and the microbial composition was determined by 16S sequence analysis. Interestingly, while the wildtype siblings housed alone harbored a significantly different microbiota compared to their IL22–/– siblings, this difference between IL22–/– and wildtype was completely lost upon co-housing. Here, the co-housed wildtype and IL22–/– mice both had lower abundance of *Lactobacillus*, *Bacteroides*, *Ruminococcus*, *Turicibacter*, *Anaerobacter, Parabacteroides,* and *Hespellia* and higher abundance of *Coprococcus, Allobaculum, Barnesiella, Alistipes, Xylanibacter, Butyricimonas*, and *Helicobacter* compared to the wildtype siblings housed alone (Zenewicz et al., 2013).

Intriguingly, the wildtype mice that were co-housed for four weeks with the IL-22–/– siblings showed a reduced expression of RegIIIγ and RegIIIβ in the colon compared to their wildtype siblings that were separately housed (Zenewicz et al., 2013). So, it seems that the microbes of IL-22–/– mice were transmitted to wildtype litter- and cage-mates, and in turn were able to influence the levels of antimicrobial peptides and DSS colitis severity in these wildtype mice. How these microbes upon transmission find a niche and are able to establish themselves in immune-competent wildtype mice, let alone subsequently have an effect on anti-microbial peptide production in these wildtype mice is as yet unclear, but warrants further investigation. Using mice conventionalized at neonatal or adult age with these 'colitis transmitting' microbes (from mice or from human IBD patients) might lead to an understanding of the underlying mechanisms.

Interestingly, it seems that IL-22 is not solely keeping bacteria clear from the epithelial surface and favoring species to colonize the gut lumen, but might also ensure lymphoid tissue colonization (Fung et al., 2016). It was shown that IL-22 derived from ILC3 cells played a role in the colonization of lymphoid tissues by certain species. By inoculating IL-22–/–, Rag–/–IL-22–/–, WT and IL17a–/– mice with one of the species that have been shown to colonize lymphoid tissues, *Achromobacter*, it was shown that the mice lacking IL-22, lacked *Acromobacter* colonization of lymphoid tissue (Fung et al., 2016). It seems, therefore, that IL-22 modulates the microbiota giving some species (able to colonize lymphoid tissue) a competitive advantage. How this permissive effect of IL-22 on microbes colonizing the lymphoid tissues in mice can be explained in the context of its clear role in maintaining epithelial barrier integrity, protecting against unwanted epithelial-attachment and translocation by increasing mucus and antimicrobial peptide production is currently unclear. This however, does link back to the previous study by Zenewicz et al. (2013), in which it was shown that IL-22–/– microbes were able to colonize wildtype cage-mates and where able to influence antimicrobial peptide production (reduced RegIIIβ and RegIIIγ) in these wildtype hosts.

In the study of Fung et al. (2016) the observed colonization of lymphoid tissue by certain bacterial species (members of the α-proteobacteria and β-proteobacteria) appeared to be important for the protection against DSS colitis by their induction of IL-10 (and IL-22). It might be interesting to study these processes early in life of the mice. Does lymphoid tissue colonization occur naturally and is IL-22 involved in this process during early natural colonization? Does this occur in other species than laboratory mice as well? For example, it might also be interesting to investigate whether this process of IL-10 induction through colonization of dendritic cells also exists in those species that lack mucosal-associated lymphoid tissues in the intestines, like fish, or whether this is an evolutionary adaptation that evolved with the development of lymph nodes draining the intestine.

IL-22 is conserved in zebrafish (Igawa et al., 2006; Siupka et al., 2014) and also found in other fish species (Corripio-Miyar et al., 2009; Monte et al., 2011; Costa et al., 2013; Yang et al., 2020). In zebrafish, expression of IL-22 was seen in the intestine and gill at baseline and increased after a challenge with TLR agonists (LPS/Poly I:C) (Siupka et al., 2014). When IL-22 was injected to mpx:GFP/lysC:DsRED2 double transgenic fish (staining myeloperoxidase-producing cells green and granulocytes red, respectively) it induced GFP expression specifically in enterocytes (Siupka et al., 2014). It has been shown that next to neutrophils, enterocytes also express myeloperoxidase during infection (Rendueles et al., 2012). Thus, zebrafish IL-22 also seems to signal to gut epithelial cells, just like mammalian IL-22. Constructing IL-22–/–, IL-22R–/–, and IL-22BP–/– zebrafish might shed light on the effect of IL-22 on epithelial barrier function in these fish.

It has been shown using zebrafish that knock-down of IL-22 by morpholino in larval zebrafish in the absence of bacterial infection, tended to increase mortality (Costa et al., 2013). During bacterial bath exposure to *Aeromonas hydrophila* or LPS, the IL-22 knock down larvae showed increased severity of disease; they displayed higher levels of *tnfa* and *il1b* expression and reduced survival (Costa et al., 2013). This might indicate that like in mammals, low levels of IL-22 are necessary to maintain homeostasis and increased levels might be necessary to combat infections.

Recently, ILC-like cells expressing *il22* have also been discovered in zebrafish using single-cell transcriptional analysis in rag1-deficient zebrafish (lacking B and T lymphocytes) (Hernandez et al., 2018). However, these cells differ from the mammalian counterparts. Specifically, zebrafish ILC-like cells were not found to constitutively express cytokine receptors

which human and mouse ILCs do express, nor AhR and pattern recognition receptors (Hernandez et al., 2018). Instead, zebrafish ILC-like cells expressed novel immune-type receptors (NITRs), putative orthologs of mammalian natural cytotoxicity receptors (NCRs) and killer cell immunoglobin like receptors (KIRs) (Hernandez et al., 2018). Thus, these ILC-like cells in zebrafish may possibly resemble human and mice NCR + ILC3s in key ways, including IL-22 expression in response to bacterial infection. Nevertheless, these aforementioned receptor differences might have consequences for their regulation.

Besides ILC-like cells, other potentially conserved relevant factors regarding IL-10 and IL-22 signaling have also been discovered in the zebrafish genome, strengthening the comparisons that can be made between these fish and mammals. One such factor involved in IL-22 signaling is IL-23, that acts via STAT3 to stimulate IL-22 production in ILCs and T cells (Zheng et al., 2008; Guo et al., 2014). Both *il23a* (p19) and *il23r* have been annotated in the zebrafish genome as well as *il1b*. IL-23a and il1b expression has been shown to be upregulated in zebrafish colitis (Brugman et al., 2009; Oehlers et al., 2012). Whether *il23* and *il1b* induce *il22* production in zebrafish needs to be determined.

A word of caution in light of similarity of genes or cells of different species is worth giving here. As for similarity of genes between (host) species, it has to be noted that (automatic) annotation might not mean functional conservation as has been shown for the IL-10R2 (Piazzon et al., 2016). In terms of receptor similarity it is shown that for IL-10R1 functional homology exists between fish and mammals. Annotation of the IL-10R2 (the receptor that, when conserved, also might bind IL-22) has been confusing. Since the two putative IL-10R2 receptors (CRFB4 and CRFB5) are very similar in protein structure and genomic organization both are annotated in the zebrafish database[1] as IL-10R2 (ENSDARG00000078042 and ENSDARG00000068711). Studies performed in grass carp actually showed that CRFB4 is probably the functional homolog of IL-10R (Wei et al., 2014; Piazzon et al., 2016). Additionally, the lack of cell markers for specific cell types in zebrafish sometimes leads to assumptions on the presence of certain cell types only on the basis of gene expression data. This should always be interpreted with caution.

The presence of IL-22BP in zebrafish has been predicted via phylogenetic analysis (Stein et al., 2007) and its cDNA has been cloned (Levraud et al., 2007). Recently, IL-22BP has been functionally characterized in mandarin fish *Siniperca chuatsi* (Huo et al., 2019). In this study, IL-22BP bound to IL-22 and thereby prevented the induction of target genes of IL-22. This suggests that at least in mandarin fish, IL-22 and IL-22BP are functionally conserved. However, whether IL-22 has the same microbiota modulating effects in (zebra)fish as in mammals during homeostasis needs to be further investigated.

In conclusion, studies performed in IL-22–/– mice are clearly showing microbiota modulating potential of IL-22. IL-22 induces antimicrobial activities, mucin production and increasing epithelial integrity, thereby preventing microbes to adhere and invade epithelial cell surfaces. However, at the same time IL-22 seems to allow certain bacteria to colonize lymphoid tissues necessary for IL-10 regulatory processes, by currently unknown mechanisms. Additionally, it is unclear why IL-10R2–/– mice do not show differences in their microbial composition, while this receptor subunit is also part of the IL-22R (IL-22R1, IL-10R2) and knock out of this subunit should also impair IL-22 signaling, shown to play a role in shaping the microbial composition. For example, Paneth cell specific IL-22R(1)–/– mice also showed an altered microbial composition (Gaudino et al., 2021). The fact that the IL-22Rα (IL-22R1) subunit also binds IL-20, IL-22, and IL-24, makes interpretation of these studies all the more complicated. Next to this, the role of IL-22BP might also be important in regulating IL-22 function, but it is unclear whether binding of IL-22 to IL-22BP has any effect on the microbiota-regulating properties of IL-22. Investigating changes in microbial composition in IL-22–/– animal models from birth or start of microbial environment exposure might shed more light on the establishment of beneficial microbial communities in the presence or absence of IL-22.

CONCLUSION AND FUTURE PERSPECTIVES

As has become clear from recent advances in our understanding of host microbe interactions, the interplay between the environment, host and colonizing microbes involves complex and dynamic processes. In **Figure 1** a schematic overview of IL-10 and IL-22 microbiota modifying effects is given. Early in life, bacterial species colonize the host. The host subsequently mounts both inflammatory and regulatory responses, in turn influencing the microbial composition. IL-22 has been convincingly shown to affect the microbiota composition and appears to create an environment that allows certain microbes to get the competitive advantage and even colonize host lymphoid tissue (resulting in IL-10 production). The exact mechanisms by which IL-22 enables some bacteria to colonize lymphoid tissues, whereas on the other hand is necessary to prevent epithelial-attachment via antimicrobial peptide induction and induction of mucus production, is currently unclear. For IL-10 the microbiota modulating potential is not so clear. The difficulty in comparing the different studies stems from the variation in co-housing and the use of littermates, as well as the timing of the experiments. IL-10–/– mice develop enterocolitis over time, making it difficult to address whether dysbiosis precedes or follows the inflammatory process. Detailed investigation into the first neonatal colonization processes might shed some light on the role of microbial induction of these regulatory cytokines (and cell types induced), as well as the effect of the induction of these regulatory cytokines on shaping the microbial community. This also holds true for the use of germfree models. Most studies compare germfree mice with wildtype mice at adult age. Likewise, when colonization effects are studied, mostly adult germfree mice are used, which might obscure those processes that are limited to (or play a bigger role) in early life. Although germfree mice have not experienced microbial colonization (and in that sense are naive) this does not mean that several other developmental processes are naive in their ability to respond to environmental stimuli.

[1] ensembl.org

There might be a specific developmental window ('a critical period in development in which an organism's phenotype may be influenced by intrinsic and extrinsic factors,' Burggren and Mueller, 2015) by which certain host processes can be influenced by the microbiota. Indeed, by comparing transcriptional profiles of intestinal regions of conventionalized (at adult age) and conventionally raised (from birth) germfree mice it was shown that the gene signature that distinguishes conventionally raised from adult conventionalized mice was region-specific as well as age-dependent (El Aidy et al., 2013b). Therefore, succession of microbial species (from pioneers to stable, yet dynamic, microbiota) in interaction with the host, might also influence host immunity in early life, while not being apparent at later stages. A deeper understanding on which immune cells and pathways can only be influenced during this window of opportunity early in life, and those that can be modified throughout life might help develop better targeted dietary (prebiotic) or probiotic intervention studies.

The processes under homeostatic conditions (more the focus of this review) might be very different from those in the context of inflammation. It has been reported for example, that IL-10–/– mice exhibited enhanced IL-22 responses toward *Clostridium difficile* infection (Cribas et al., 2021). Moreover, recently it was shown that spontaneous colitis in IL-10–/– mice is dependent on IL-22; IL-10–/–IL22–/– mice did not display spontaneous colitis (Gunasekera et al., 2020). IL10–/–IL-22–/– mice showed enhanced frequencies of Th17 cells, just like IL-10–/– mice, while microbial diversity in IL-10–/–IL-22–/– mice was restored (compared to IL10–/– mice) and no epithelial hyperplasia nor increased RegIIIγ was seen (Gunasekera et al., 2020).

Furthermore, IL-10 and IL-22 responses are shown to be dynamic and probably dependent on the location within the intestines and the life stage of the host. Indeed, it has been shown that in the ileum of wildtype mice ILC3 IL-22 expression increased until 4 weeks of life after which levels declined. This decrease after 4 weeks of age was not shown in Rag1–/– mice (lacking adaptive immune cells). Further investigation showed that both regulatory T cells and Th17 cells could diminish ILC3 activation by decreasing IL-23 production or regulation of the bacterial burden respectively (Mao et al., 2018). Thus it seems that early in life ILC3s shape the murine ileal microbiota through IL-23 and IL-22 dependent mechanisms, which is dampened with the development of adaptive immunity, when other mechanisms of host–microbiota cross-talk take over (Mao et al., 2018).

Differentiating between innate and adaptive sources of IL-10 and IL-22 is a challenge. Here, the zebrafish model might offer a solution. Since zebrafish develop *ex utero*, the early development of the immune system can be studied *in vivo* in a live animal using transgenic reporter zebrafish. Innate immunity develops from 2 dpf and adaptive immunity develops from 10 dpf onward (at this time CD4 + lymphocytes have been shown to leave the thymus). IL-22 and IL-10 seem to be conserved, although their regulation may differ (Costa et al., 2013; Siupka et al., 2014; Piazzon et al., 2016).

Furthermore, although most studies have focused on bacterial species interacting with the host, we now know that fungi (including yeasts), viruses/bacteriophages and protozoa should also be taken into account. Interestingly, viruses express IL-10 mimics influencing host responses (for review: Piazzon et al., 2016). Analysis of this intricate interplay between members of different kingdoms in the developing host is still in its infancy and predicts an exciting and challenging future for researchers. Focusing on the early life window and understanding possible long-term effects of microbial colonization on host immune development and general host health might enable smart targeted therapies to prevent chronic diseases later in life.

AUTHOR CONTRIBUTIONS

EK wrote the manuscript and made the table. MK wrote and edited the manuscript. SB wrote and edited the manuscript, made the figure, and secured the funding. All the authors contributed to the article and approved the submitted version.

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Lactobacillus reuteri Alleviates Gastrointestinal Toxicity of Rituximab by Regulating the Proinflammatory T Cells *in vivo*

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Rituximab (RTX) is a widely used anticancer drug with gastrointestinal side effects, such as nausea, vomiting, and diarrhea. The reason for these side effects is still poorly understood. Previous studies have reported that the intestinal microbiota is associated with the occurrence of disease and the therapeutic effect of drugs. In this study, we observed mucosal damage, inflammatory cell infiltration and increased intestinal inflammatory factor expression in RTX-treated mice. RTX also changed the diversity of the intestinal microbiota in mice, and decreased abundance of *Lactobacillus reuteri* was observed in RTX-treated mice. Further experiments revealed that intragastric administration of *L. reuteri* in RTX-treated mice attenuated the intestinal inflammatory response induced by RTX and regulated the proportion of helper T (Th) cells. In conclusion, our data characterize the effect of the intestinal microbiota on RTX-induced intestinal inflammation, suggesting that modifying the gut microbiota may represent a positive strategy for managing adverse reactions.

Keywords: rituximab, intestinal microbiota, *Lactobacillus reuteri*, gavage methods, gastrointestinal toxicity

INTRODUCTION

Although there are many new methods for cancer treatment, drug therapy for cancer is still an important and indispensable treatment modality that is mainly based on the ability of drugs to clear cancer cells, reduce tumor growth and relieve pain. Anticancer drugs kill tumor cells by specifically recognizing surface markers on tumor cells and stimulating the immune response. For example, rituximab binds to the CD20 antigen on B-cell-derived tumor cells to initiate an immune response that mediates B-cell lysis. Possible mechanisms of B-cell lysis include complement-dependent cytotoxicity (CDC) and antibody-dependent cytotoxic (ADCC) effects. These killing effects are not specific to tumor cells and also affect B cells, T cells, and regulatory T cells. Infection and gastrointestinal toxicity often occur during the course of treatment (El Fassi et al., 2008; Agathocleous et al., 2010; Ardelean et al., 2010; Blombery et al., 2011; Sekkach et al., 2011; Lipka et al., 2016; Shahmohammadi et al., 2018; Kaegi et al., 2019), and they can treatment efficacy and

patient compliance to a certain extent. However, there is currently no effective treatment to reduce gastrointestinal mucosal lesions. After proliferation and differentiation, crypt cells migrate to the villi of the small intestine to replace the exfoliated mature epithelial cells and become intestinal mucosal epithelial cells (Farrell et al., 1998; Bau et al., 2016). Some drugs kill crypt stem cells of the small intestine, damaging the absorption and barrier function of the intestinal mucosa and resulting in gastrointestinal tract toxicity and side effects. Some studies have shown that in cancer chemotherapy, the incidence of intestinal mucositis is as high as 40% (Boukhettala et al., 2009; Beutheu et al., 2014). An increasing number of studies have shown that the intestinal microbiota is involved in regulating the efficacy and toxicity of chemotherapy drugs (Li et al., 2016, 2017; Wardill and Tissing, 2017; Wilson and Nicholson, 2017; Zimmermann et al., 2019).

There are a large number of microbial communities in the intestine, including approximately 3×10^{13} bacteria (Sender et al., 2016). Most of them coexist with the host in a dynamic balance. However, when the health status of the host is affected, the composition of intestinal flora and the abundance of certain bacteria change. Expansion of some symbiotic bacteria may lead to more serious effects, while the reduction of some symbiotic bacteria will cause the opposite effects; for example, Routy et al. (2018) found that patients with high *Akkermansia muciniphila* abundance tend to experience improved efficacy after receiving PD-1 therapy (Zhou et al., 2018). Intestinal microbes even play an important role in the health of the host by regulating the immune response or the well-known "liver–brain–gut" axis circulation. When the tissue is damaged after infection or injury, inflammatory cells enter the intestine from the systemic circulation or intestinal cistern to restore the body's balance (Zhou et al., 2018). Therefore, intestinal injury caused by RTX may cause an imbalance in enteric cells, which may lead to malignant inflammatory circulation.

It should be noted that a large number of inflammatory cells in the intestine can also affect the intestinal microbiota. In addition, some drugs can affect the intestinal microbiota. For example, Viaud et al. found that after 7 days of cyclophosphamide treatment, the total number of bacteria in the small intestine of mice did not decrease, but the abundance of *Lactobacillus* and *enterococci* decreased. Moreover, after drug treatment, the villi of the small intestine in mice were shortened, the intestinal barrier was damaged, and inflammatory cells had aggregated (Viaud et al., 2013). Moreover, specific gram-positive bacteria (*Lactobacillus johnsonii* and *Enterococcus hirae*) regulate the accumulation of cyclophosphamide-driven Th1 and Th17 cell responses (Viaud et al., 2013). It has been reported that CTLA4 antagonists induce T cell-mediated intestinal mucositis, which is related to changes in the intestinal flora (Viaud et al., 2013; Authors et al., 2015; Sivan et al., 2015; Vétizou et al., 2015). These studies demonstrate that homeostasis of the intestinal flora is important for the host immune system and intestinal epithelium. In our study, we identified the effect of rituximab on intestinal mucosal injury, the changes in different immune cells in mesenteric lymph nodes, and the resulting changes in intestinal microbiota, which are associated with the development of intestinal mucositis. We also found that this toxicity is attenuated by intragastric administration of specific Lactobacillus species. Our aim was to characterize these changes and examine the effects of altering the intestinal microbiota on rituximab-induced mucositis. These findings may contribute to subsequent studies on methods to attenuate the gastrointestinal toxicity of certain drugs.

MATERIALS AND METHODS

Animal Experiments

Female BALB/c mice (6–8 weeks old) were purchased from Beijing Vital River Laboratory Animal Co., Ltd. (Beijing, China). All animals were housed in a pathogen-free environment and maintained at 22 ± 2°C under 12-h light/dark cycle conditions. All animal experiments were performed according to the guidelines approved by the Animal Protection Committee of Sichuan University (Sichuan, China, ID: 2018091815).

Two experiments were performed to investigate the relationship between rituximab toxicity and the intestinal microbiota. In the first experiment, female BALB/c mice were divided into two groups. Mice in the experimental group were intraperitoneally injected with 4 mg rituximab (Roche, 100 mg/10 ml), while mice in the control group received sterile phosphate balanced solution (PBS). In the second experiment, female BALB/c mice were randomly divided into six groups with six mice per group. Mice in the antibiotic (ATB) group, ATB + RTX group and ATB + RTX + *Lactobacillus reuteri* group were treated with metronidazole (1 g/L, Solarbio 443-48-41) supplied in the drinking water on day −7 for 5 days that was then stopped for 2 days. On day 0, mice in the RTX and ATB + RTX groups were intraperitoneally injected with rituximab at 4 mg/mouse. Mice in the RTX + *L. reuteri* and ATB + RTX + *L. reuteri* groups were simultaneously gavaged with 1×10^8 CFU of *L. reuteri* in 200 μl PBS for 7 days. The other two groups (ATB and Con) were gavaged with sterile PBS. All mice were sacrificed on day 7.

Bacterial Culture

Lactobacillus reuteri (ATCC 53608) was purchased from American Type Culture Collection (ATCC) and were cultured in De Man, Rogosa and Sharpe (MRS) broth medium (M264-02) at 37°C and under anaerobic conditions. The bacterial suspension was washed with sterile PBS, centrifuged (3,200*g*, 5 min) and resuspended in PBS until OD_{600} = 1, which was approximately 1×10^8 colony forming units (CFUs)/ml (Jiménez-Flores et al., 2010).

Stool Sample Collection and DNA Extraction

According to different animal experimental schemes, stool samples were collected from mice on days −7, 0, and 7 during the first experiment and immediately stored at −80°C. In the second animal experiment, fecal samples were collected from mice on days −7, −5, 0, and 7 and were immediately stored at −80°C until processing with a fecal DNA isolation kit (Foregene Co.,

Ltd., Sichuan, China). Samples were stored at −80°C according to the fecal DNA isolation kit manufacturer's instructions.

16S Sequencing and Bioinformatics Analysis

The V4 region of 16S rDNA was amplified by Beijing Novogene Technology Co., Ltd., and sequenced on the NovaSeq6000 platform. Paired-end reads were merged using FLASH (V1.2.7)[1] (Magoč and Salzberg, 2011), a fast and accurate analysis tool that was designed to merge paired-end reads when at least some of the reads overlapped reads generated from the opposite end of the same DNA fragment, and the splicing sequences were represented raw tags. Quality filtering of the raw tags was performed under specific filtering conditions to obtain high-quality clean tags (Bokulich et al., 2013) according to QIIME (V1.9.1).[2] Tags were subsequently compared with the reference database (Silva database)[3] using the UCHIME algorithm (UCHIME algorithm)[4] (Edgar et al., 2011) to detect chimeric sequences, which were then removed (Haas et al., 2011), yielding the effective tags.

Sequence analysis was performed using Uparse software (Uparse v7.0.1001)[5] (Edgar, 2013). Sequences with ≥97% similarity were assigned to the same OTUs.

Representative sequences for each OTU were screened for further annotation. For each representative sequence, the Silva Database (see text footnote 3) (Quast et al., 2013) was used based on the Mothur algorithm to annotate taxonomic information. To study the phylogenetic relationship of different OTUs and the difference in the dominant species in different samples (groups), multiple sequence alignment was conducted using MUSCLE software (Version 3.8.31)[6] (Edgar, 2004).

Linear discriminant analysis (LDA) and effect size (LEfSe) analysis methods (Segata et al., 2011) used the Kruskal-Wallis test to compare the abundance of all bacterial clades between day 0 and day 7 at a predefined α of 0.05. Significantly different vectors obtained by comparing abundance among the groups were used as inputs for LDA, resulting in an effect size. Compared with traditional statistical tests, the main advantage of LEfSe is that in addition to the *p*-value, LEfSe also produces an effect size. This feature allows us to sort the results of multiple tests based on the size of the difference between groups.

Quantitative Real-Time PCR

Quantitative real-time PCR (qPCR) was used to quantify bacterial 16S rDNA gene abundance in feces samples. Primers targeting the rDNA gene of *L. reuteri* (F: ACCGAGAACACCGCGTTATTT, R: CATAACTTAACCTAAACAATCAAAGATTGTCT) (Rothhammer et al., 2016) and primers targeting β-actin (F: CCCAGGCATTGCTGACAGG, R: TGGAAGGTGGACAGTGAGGC) were used.

Total RNA was extracted from cells or small intestine segments of mice using a kit (Foregene, China) and was quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). According to the manufacturer's instructions, cDNA was synthesized using a PrimeScript RT Kit with gDNA Eraser (Vazyme, China). Transcription levels of TNF-α, IL-1β, IL-6, IL-10, claudin-1, and β-actin were analysed by SYBR Green PCR Master Mix (Vazyme, China) on a CFX96 real-time system (Bio-Rad). The relative expression was calculated by ΔCT method. The sequences of the gene-specific primers were as follows: TNF-α (F: CCCAGGGACCTCTCTCTAATC, R: ATGGGCTACAGGCTTGTCACT); IL-1β (F: ATCTCGCAGCAGCACATCAA, R: ACGGGAAAGACACAGGTAGC); IL-6 (F: CCAGTTGCCTTCTTGGGACT, R: GTCTCCTCTCCGGACTTGTG); IL-10 (F: CATCGATTTCTTCCCTGTGAA, R: TCTTGGAGCTTATTAAAGGCATTC); and claudin-1 (F: TGCCCCAGTGGAAGATTTACT, R: CTTTGCGAAACGCAGGACAT).

Quantitative real-time PCR was performed on a CFX96 Real-time system (Bio-Rad) under the following cycling conditions: 95°C for 30 s, 35 cycles at 95°C for 10 s, and 60°C for 30 s. Then, the default melting curve acquisition program was used.

Hematoxylin and Eosin Staining for Histological Analyses

The mice were sacrificed 1 week after RTX treatment. Ileum and colon samples were fixed in 4% PFA for at least 48 h. Subsequently, the tissues were cut into small pieces, embedded in paraffin and cut into 4-μm sections that were stained with hematoxylin and eosin (H&E) and scored by pathologists. In the semi-quantitative analysis of histology, villus atrophy, necrosis, and inflammatory cell infiltration scores were as follows: 0 (no injury), 1 (mild injury), 2 (moderate injury), and 3 (severe injury). The lengths of 10 intact and well-oriented villi were measured using ImageJ software (Yu et al., 2019).

Flow Cytometry Experiments

Monoclonal antibodies against mouse CD3E, CD4, Foxp3, IL-4, CD19, IL-10, IFN-γ, IL-17A, CD11b, CD11c, F4/80, CD206, and CD16/32 were purchased from BD Biosciences. All mice were sacrificed at the end of the experiment. Mesenteric lymph nodes (MLNs) and spleens were crushed in a cell filter (75 μm nylon; BD Falcon), and the cell suspension was treated with erythrolysis buffer (Beyotime Biotechnology) at room temperature for 5 min. Then, the cells were washed three times and suspended in PBS. For surface antibody staining, the cell suspension was combined with various antibodies and incubated in the dark at 4°C for 30 min. To stain for Foxp3, IL-4, IL-10, IL-17A, and IFN-γ, the cells were fixed in 4% neutral paraformaldehyde after surface marker staining, treated with 0.5% Triton-100 (PBS dilution) at 4°C for 20 min, washed again, and then incubated with corresponding antibodies. Flow cytometry (FACSCalibur; BD Biosciences) was used to obtain the cell events, and the data were analysed with NovoExpress software.

[1] http://ccb.jhu.edu/software/FLASH/

[2] http://qiime.org/scripts/split_libraries_fastq.html

[3] https://www.arb-silva.de/

[4] http://www.drive5.com/usearch/manual/uchime_algo.html

[5] http://drive5.com/uparse/

[6] http://www.drive5.com/muscle/

Cell Culture and Stimulation With *Lactobacillus reuteri in vitro*

The mesenteric lymph node (MLN) cells of mice were cultured at 37°C and 5% CO_2. Ten percent fetal bovine serum (Gibco, United States) was added to RPMI 1640 cell culture medium. For *L. reuteri*'s anti-inflammatory test, MLN cells were cultured in a 6-well plate at a density of 2×10^6 cells per well and then co-incubated with *L. reuteri* (infection multiple = 10, 2×10^6 cells and 2×10^7 CFU) in the presence or absence of LPS (10 ng/mL, PrimeGene) (Chae, 2018) for 4 h. After incubation, cells were collected for gene expression analysis.

Statistical Analysis

All data were statistically analysed using GraphPad Prism 8 and are shown as the mean value ± SEM of the mean. Statistical significance was evaluated at a level of $P < 0.05$ using unpaired two-way analysis of variance (ANOVA) or *t*-test analysis, * indicated significance at $p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}P < 0.0001$, respectively.

RESULTS

Rituximab Induces Intestinal Mucositis in Mice

To investigate the intestinal toxicity of RTX, mice were treated with a single dose of RTX (4 mg/mouse, i.p.) for 7 days (**Figure 1A**). Compared with the control group, RTX caused significant weight loss in mice (**Figure 1B**). In contrast to the control group, villous atrophy and intestinal epithelial injury of mice in the RTX treatment group were obvious, as shown in **Figure 1C**. Moreover, obvious inflammatory cell infiltration was apparent in the colon tissue of RTX-treated mice (**Figure 1C**). Compared with the control group, the intestinal mucositis score (**Supplementary Figure 2**) in the RTX group

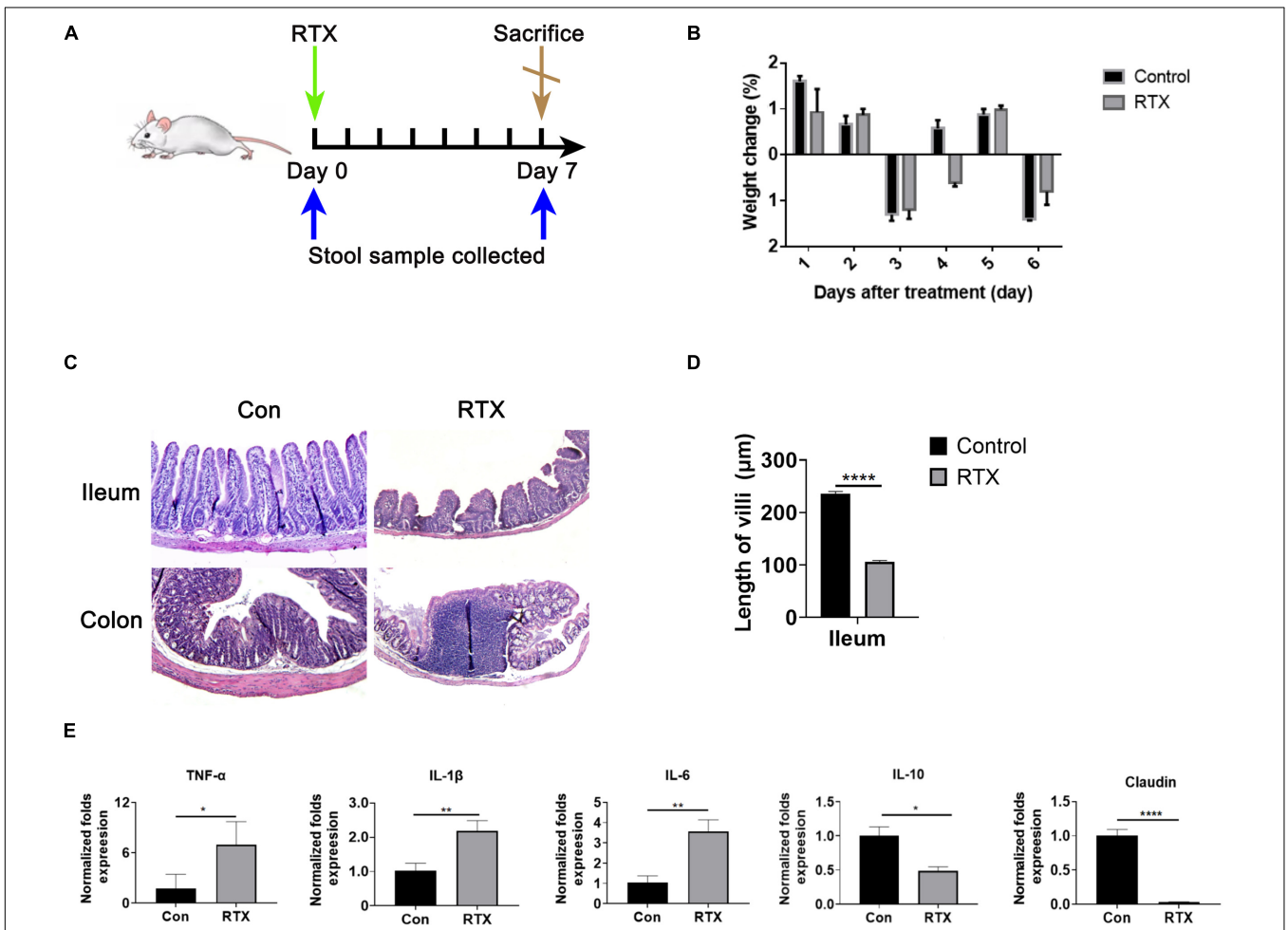

FIGURE 1 | Rituximab (RTX) causes marked intestinal mucositis. **(A)** Schematic diagram of RTX administration and stool sample collection. BALB/c mice were intraperitoneally injected with RTX or normal saline on day 0 and then sacrificed on day 7. Feces were collected before and after treatment. **(B)** From day 0 to day 7, the body weights of mice in the control and RTX treatment groups were changed. **(C)** H&E staining of the ileum and colon (magnification, 40×). **(D)** Villus length of ileum and colon. **(E)** Expression levels of TNF-α, IL-1β, IL-6, IL-10, and claudin in the ileum of mice treated with RTX or normal saline. Data are expressed as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}P < 0.0001$.

was significantly increased ($P < 0.001$; **Figure 1D**). These results suggest that RTX treatment causes intestinal damage and inflammatory cell infiltration. To further verify the intestinal inflammatory response induced by RTX, qPCR was used to assess the expression of inflammatory markers in the ileum. We observed that expression levels of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 in the ileum of RTX-treated mice were significantly increased, while expression of IL-10 was significantly decreased (**Figure 1E**), the colon inflammatory factor data in the **Supplementary Figure 3**. Claudin-1 is a tight junction transmembrane protein that constitutes tight junctions between cells. It functions mainly to regulate the permeability of the barrier structure. The stability of tight junctions is related to the complex interactions between claudin proteins and other tight junction proteins. Our results showed that compared with the control group, the expression of claudin in the intestinal tissue of RTX-treated mice was significantly decreased ($P < 0.0001$; **Figure 1E**), which may increase intestinal permeability, cause bacterial migration to the site of intestinal injury, and further promote the local inflammatory response.

Rituximab Induces Alterations in T Cell Subsets

We observed that compared with the control group, the infiltration of $CD4^+$ dendritic cells in intestinal tissues were significantly increased after RTX treatment (**Supplementary Figure 1**), and $CD4^+$ cells were subdivided into subsets of T helper (Th), such as Th1, Th2, Th9, Th17, regulatory T (Treg) cell, and follicular helper T cell subtypes. Our results indicated that compared with the control group, levels of Th1 ($CD4^+$/IFN-γ^+) and Th17 cells ($CD4^+$/IL-$17A^+$) in the MLN of RTX-treated mice were significantly increased (**Figure 2**). Th1 cells promote the cell-mediated inflammatory response by inducing the activation of macrophages, NK cells, and B cells (Carrasco and Fernández-Bañares, 2017). Most of the effector capacity of Th17 cells comes from the fact that IL-17, together with TNF-α, strongly promote inflammation by inducing the expression of adhesion molecules, proinflammatory cytokines (e.g., IL-6, GM-CSF, and G-CSF), chemokines, prostaglandin E2 and matrix metalloproteinases (Kumawat et al., 2013; Rauber et al., 2017). Th17 cells respond to infection by extracellular bacteria and fungi. Elevated Th17 cells have been found in patients with atopic dermatitis, Crohn's disease, psoriasis, and multiple sclerosis.

In addition, macrophages can adopt specialized functional phenotypes in response to various signals, known as classic activated macrophages ($CD11b^+F4/80^+$) and selectively activated macrophages ($CD11b^+CD206^+$) (Mantovani et al., 2002; Sica and Mantovani, 2012). $CD11b^+F4/80^+$ macrophages secrete proinflammatory cytokines, present antigens, promote the Th1 response, and mainly participate in the positive immune response. In contrast, $CD11b^+cd206^+$ macrophages play an immunomodulatory role by producing inhibitory cytokines, such as IL-10 or TGF-β, and downregulating the immune response (Sica and Bronte, 2007; Martinez and Gordon, 2014; Acharya et al., 2020). Compared with the control group, the content of $CD11b^+F4/80^+$ macrophages in the spleen and MLN were increased after RTX treatment, while $CD11b^+CD206^+$ macrophages were slightly decreased (**Supplementary Figure 1**). In addition, the ratio of $CD11b^+F4/80^+$/$CD11b^+CD206^+$ cells in the RTX treatment group was significantly increased, suggesting that RTX promotes the polarization of inflammatory macrophages induced by $CD11b^+F4/80^+$ cells in mice, inducing intestinal inflammation (**Supplementary Figure 1**).

Rituximab Induces Changes in Intestinal Microbial Components

Recent studies have shown that some drugs cause changes in the intestinal microbial composition, such as cyclophosphamide and carboplatin. In addition, the intestinal microbiota is closely related to drug resistance, efficacy, and toxicity (Alexander et al., 2017; Bullman et al., 2017; Gopalakrishnan et al., 2018; Matson et al., 2018; Routy et al., 2018; Zhou et al., 2018). To determine whether RTX has an effect on the intestinal flora of mice, 16S ribosomal DNA (rDNA) sequencing was used, collecting stool samples before and after RTX treatment for bacterial microbiota profiling. RTX treatment changed the microbial population in mice (**Figure 3A**). Although most OTUs were shared on day 0 and day 7, 153 OTUs were lost, 105 unique OTUs appeared after RTX treatment, and the total number of OTUs decreased. Next, we analysed the bacterial alpha diversity in the RTX treatment and control groups. We found that the total microbial diversity decreased significantly in the RTX treatment group, while no significant change was observed in the control group over time (**Figures 3B,C**). Moreover, principal component analysis (PCA) revealed that the composition of the intestinal microflora changed significantly after RTX treatment (day 7) compared with before RTX treatment (day 0) (**Figure 3D**). In the control group, there was little difference in intestinal microbiota composition between days 7 and 0 (**Figure 3D**). In addition, significant changes in the intestinal microbiota were estimated by comparing the composition of OTUs with that of normal mice. **Figure 3E** shows the increase or decrease in microbial components in response to different treatments. For example, the relative abundance of Lactobacillus decreased significantly (**Figure 3F**), while there was no significant change in these OTUs between day 0 and day 7 in the control group. In conclusion, these data suggest that RTX treatment alters the diversity and composition of the intestinal microbiota in mice (the comparison of RTX and control groups about the results of α and β diversity on day 0 and day 7 was shown in **Supplementary Figure 4**).

Identification of Key Microbial Species

To identify the most differentially abundant bacteria after RTX treatment, LDA effect size analysis (LEfSe) was performed, and we selected biomarkers with LDA scores > 4. We found that Lactobacillus was more abundant before RTX administration and decreased after 7 days (**Figures 4A,B**). We further analysed the relative abundance of different Lactobacillus species in the control and RTX treatment groups. The results showed that the most differentially abundant bacteria were *Lactobacillus gasseri* (*L. gasseri*) in the RTX treatment group, consistent with the

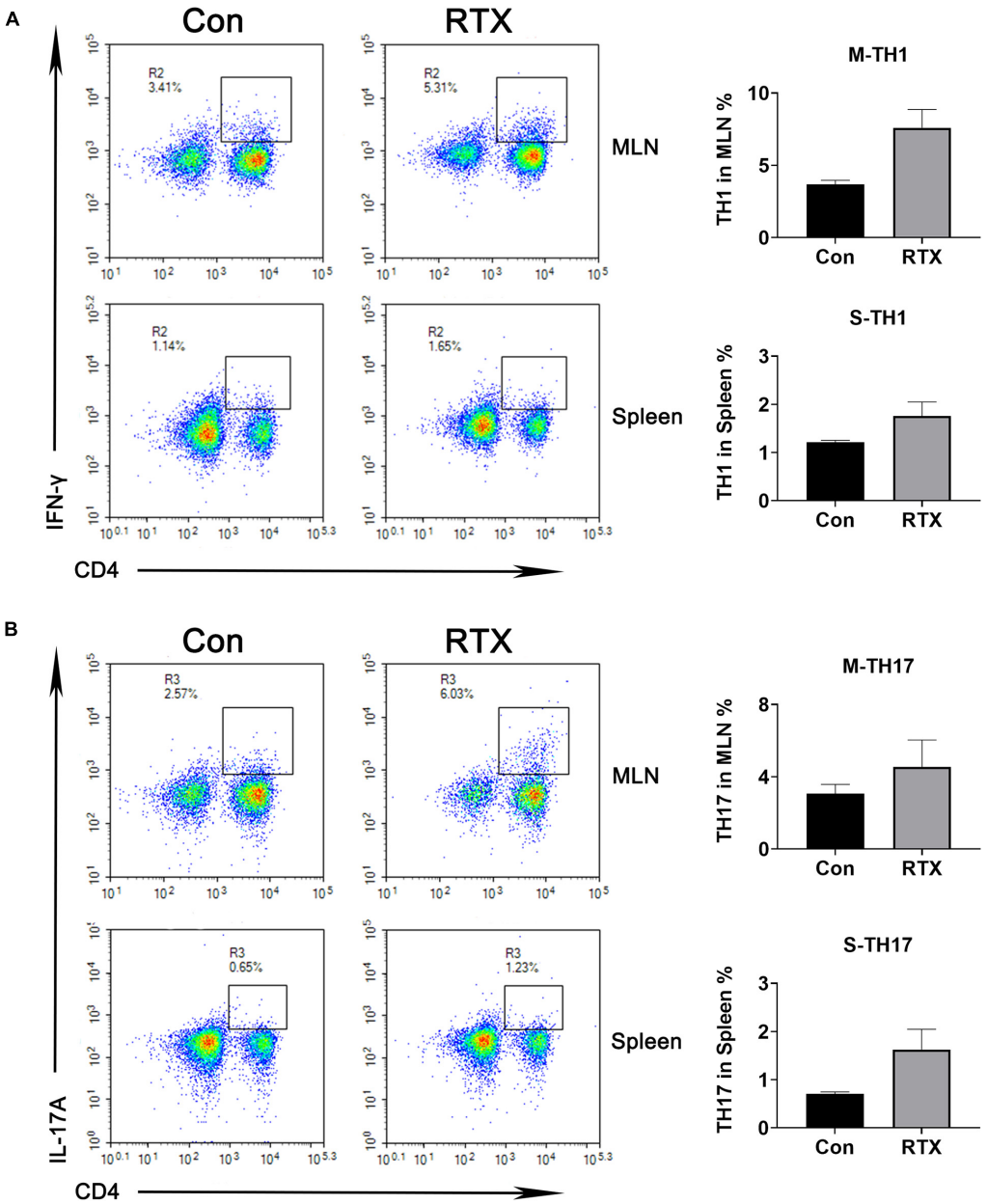

FIGURE 2 | Changes in helper T (Th) cell subsets are induced by RTX. The percentages of Th1 and Th17 cells in the spleen and MLN were detected by flow cytometry. Data are expressed as the mean ± SEM. $p < 0.05$.

control group (**Figure 4C**). Only *L. reuteri* decreased significantly in the RTX-treated group, while no significant change was observed in the control group. These results demonstrate that the number of *L. reuteri* decreased significantly after RTX treatment compared with the control group. *L. reuteri* exhibits strong adhesion to the intestinal mucosa. It improves the distribution of intestinal microbiota, inhibits the colonization of harmful bacteria and resists intestinal diseases. We conducted qPCR to quantify the number of specific Lactobacillus in feces samples. After RTX treatment, the amount of *L. gasseri* decreased, but the number in the control group also decreased, while the number of *L. reuteri* did not decrease after PBS treatment (**Figure 4D**), consistent with previous reports. It should be noted that the changes in *L. reuteri* after RTX treatment were consistent with the 16S rDNA sequencing results, suggesting that *L. reuteri* may play a role in RTX-induced intestinal mucositis.

Lactobacillus reuteri Inhibits Inflammation *in vitro*

To invaluated the role of *L. reuteri* in RTX-induced intestinal mucositis. Next, we evaluated whether *L. reuteri* inhibits

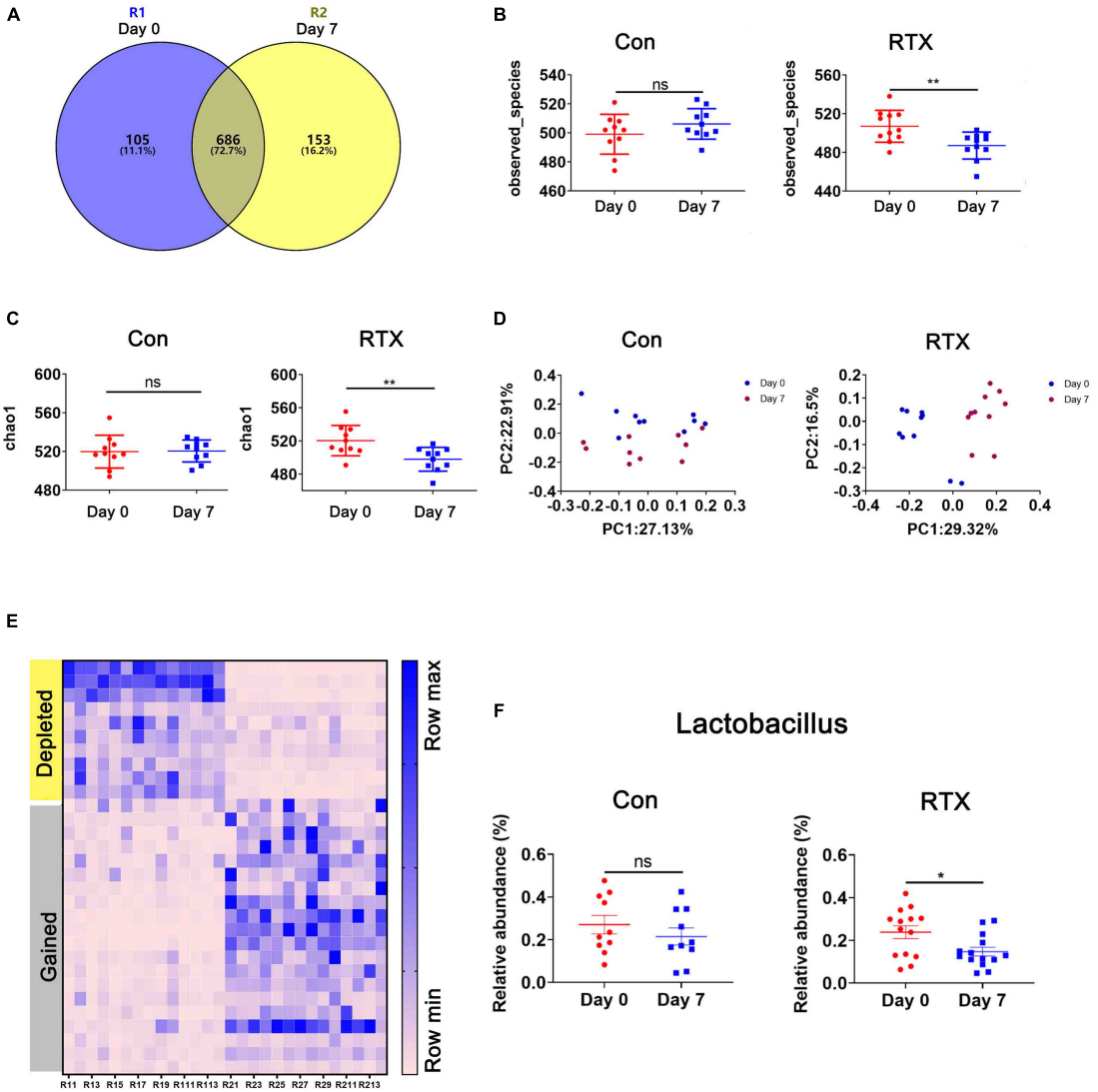

FIGURE 3 | Rituximab treatment changes the composition of the intestinal microbiota. **(A)** The number of OTUs in the intestine of RTX-treated mice on day 0 (blue), day 7 (yellow), or shared on days 0 and 7 (gray). **(B,C)** Changes in the α diversity of the intestinal microbiota in the RTX-treated and control groups are shown on days 0 and 7. **(D)** The results of principal component analysis (PCA) of intestinal microbiota in the RTX-treated group and control group on days 0 and 7. **(E)** Heatmap of OTUs of intestinal microbiota in RTX-treated mice on days 0 and 7. **(F)** The relative abundance of 2 examples of OTUs in RTX-treated mice and control mice. OTUs, operational taxonomic units. $^{*}p < 0.05$, $^{**}p < 0.01$.

inflammation *in vitro* (**Figure 5A**). Mesenteric lymph node cells of mice were cultured *in vitro* and treated with *L. reuteri* or *L. reuteri* + LPS to assess the expression of inflammatory cytokines. Inflammatory cytokine expression was increased after LPS stimulation, but the expression of inflammatory cytokines in the MLN was not upregulated in the group coincubated with *L. reuteri* (**Figures 5B–D**). These results suggest that *L. reuteri* inhibits inflammatory reactions *in vitro* to some extent.

Lactobacillus reuteri Attenuates Rituximab-Related Inflammatory Damage

It has been reported that *L. reuteri* reduces inflammatory reactions and relieves the development of colitis (Madsen et al., 1999; Liu et al., 2012; Mu et al., 2017, 2018). To verify whether the loss of intestinal *L. reuteri* leads to more severe inflammatory damage, colonic colonization was performed in antibiotic-treated mice with RTX by oral administration of *L. reuteri* (**Figure 6A**). After antibiotic treatment (day 0), levels of *L. reuteri* in the ATB, ATB + RTX, and ATB + RTX + *L. reuteri* groups were significantly decreased compared to those in the other three groups (**Figure 6B**). On day 5 after intragastric administration of *L. reuteri*, the *L. reuteri* content between the RTX + *L. reuteri* and ATB + RTX + *L. reuteri* groups was significantly higher than in the ATB, RTX, and ATB + RTX groups (**Figure 6C**). These results indicate that *L. reuteri* has a strong intestinal colonization effect in mice. Consistent with previous reports that *L. reuteri* loss exacerbates colitis, mice treated with RTX for 7 days developed more severe intestinal

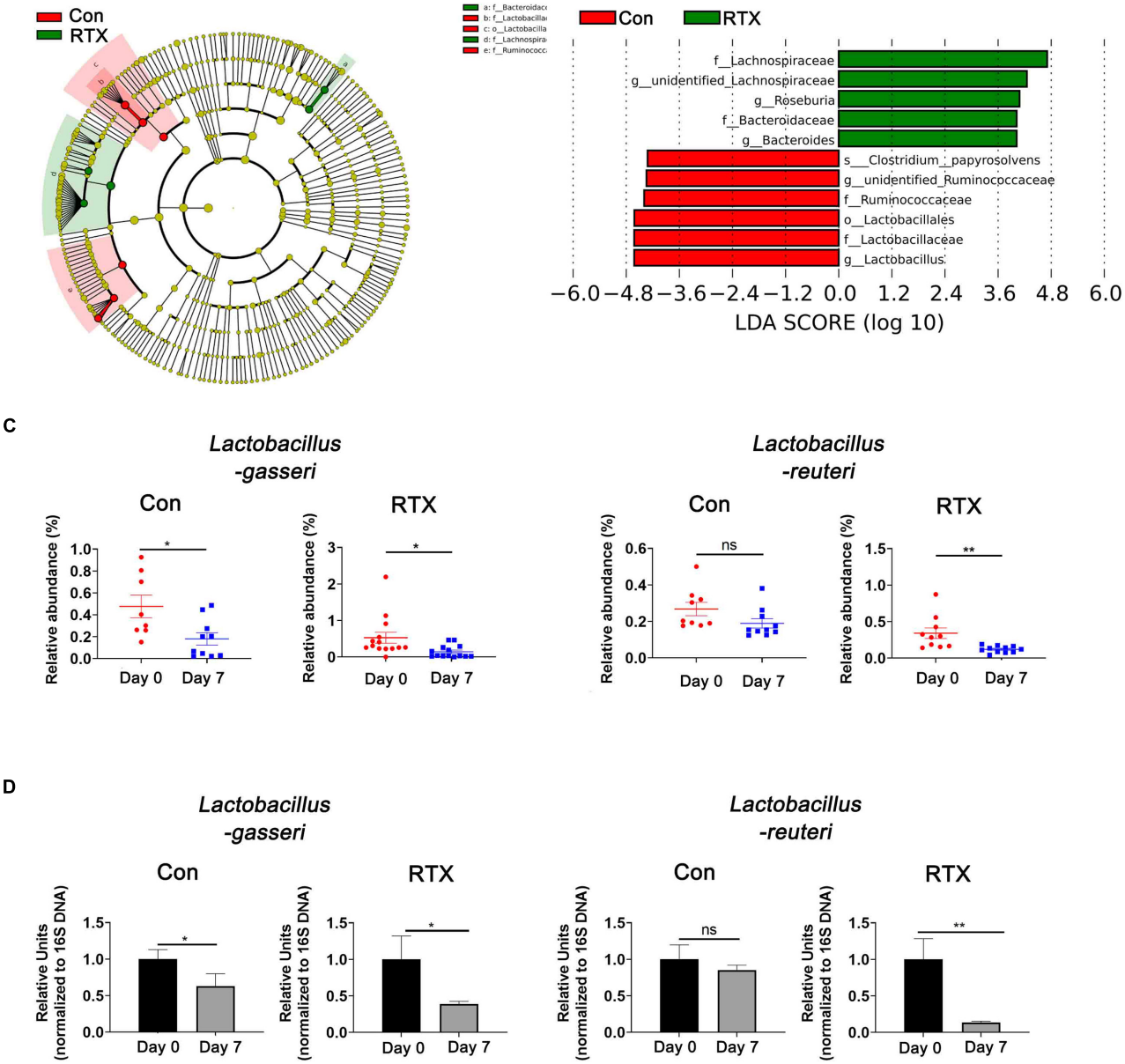

FIGURE 4 | *Lactobacillus reuteri* is associated with RTX-induced intestinal mucositis. **(A)** LEfSe analysis of differentially abundant species in RTX-treated mice on day 0 (R1) and day 7 (R2). **(B)** LDA scores of differentially abundant species in the intestine before and after RTX treatment showing only the groups with an LDA threshold > 3.6. **(C)** The relative abundance of significantly different bacteria in control and RTX treatment groups. **(D)** qPCR results of different bacteria in feces samples. Data are shown as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$.

mucositis, in contrast to the control group, villous atrophy and intestinal epithelial injury of mice in the RTX treatment group were obvious, as shown in **Figure 6D**. Moreover, obvious inflammatory cell infiltration was apparent in the ileum tissue of ATB and RTX-treated mice (**Figure 6D**) as assessed by histological analysis of increased epithelial damage (**Figure 6D**) and shortened intestinal villi of ileum (**Figure 6E**), which resulted in a reduced mucositis score (**Supplementary Figure 2**) compared with the RTX-treated control group (**Figure 6E**). In addition, compared with RTX-treated mice, *L. reuteri*-colonized mice exhibited decreased levels of inflammatory cytokines (**Figure 6F**) and increased levels of intestinal tight junction proteins (**Figure 6F**). These findings suggest that *L. reuteri* alleviates RTX-induced intestinal mucositis and reduces damage to the intestinal mucosal barrier system. Next, we analysed changes in the immune cell population 7 days after *L. reuteri* was implanted. Compared with the control group, the number of Th1 and Th17 cells in the MLN of RTX-treated mice was significantly

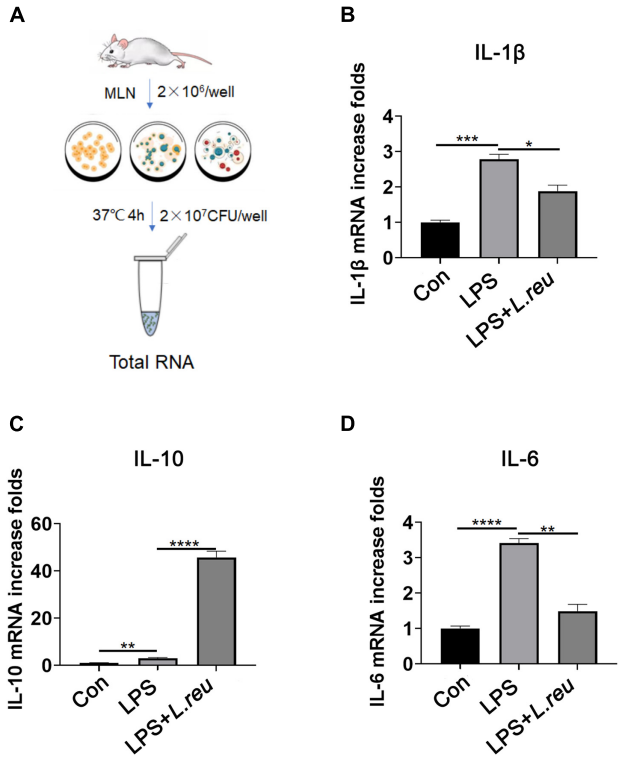

FIGURE 5 | Effect of *Lactobacillus reuteri* on the expression of IL-1β, IL-10, and IL-6 in mesenteric lymph node cells stimulated by LPS *in vitro*. **(A)** Schematic diagram of experiment setup. In the presence or absence of *L. reuteri*, MLN cells were stimulated with LPS alone for 4 h. Cells were collected for qPCR analysis. **(B–D)** mRNA expression of IL-1β, IL-10, and IL-6 in MLN cells. Data are expressed as the mean ± SEM. $p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}P < 0.0001$. Con, control; *L. reu*, *Lactobacillus reuteri*.

increased (**Figure 7A**). Consistent with our previous results, RTX treatment resulted in a significant increase in Th1 and Th17 cells in the MLN (**Figures 7A,B**). However, the number of Th1 and Th17 cells in the MLN of mice fed *L. reuteri* was significantly reduced, and Th2 cells was increased (**Supplementary Figure 5**). In addition, RTX treatment resulted in a decrease in Breg cells, and intragastric administration of *L. reuteri* resulted in an increase in Treg cells (**Supplementary Figure 5**) Breg cells in the MLN (**Figure 7C**). These results suggest that *L. reuteri* could alleviate the intestinal inflammation induced by RTX through regulating the amount of Th1 and Th17 cells.

DISCUSSION

The toxicity of many anticancer drugs seriously affects their therapeutic effects and the quality of life in cancer patients (El Fassi et al., 2008; Agathocleous et al., 2010; Ardelean et al., 2010; Blombery et al., 2011; Sekkach et al., 2011; Lipka et al., 2016; Ziętarska et al., 2017; Maestá et al., 2018; Shahmohammadi et al., 2018; Kaegi et al., 2019). Some drug treatments lead to intestinal mucosal dysfunction, including endothelial and epithelial cell death and even mucosal immune system activation (Chaveli-López and Bagán-Sebastián, 2016; de Melo Manzi et al., 2016; Shimamura et al., 2018). At present, there is no successful method to treat mucositis. In recent years, studies have shown that the intestinal microbiota regulates the therapeutic effect of some anticancer drugs (Authors et al., 2015; Sivan et al., 2015; Routy et al., 2018), but little is known about the regulatory function of the intestinal flora in the gastrointestinal toxicity of these drugs (Zhou et al., 2018; Yu et al., 2019). The intestinal mucosa acts as a selective barrier between the body and the external environment that blocks harmful substances from entering the systemic circulation but also ensures the absorption of nutrients (Isaacs-Ten et al., 2020). The intestinal microbiota lives on the layer of mucus secreted by intestinal goblet cells. The intestinal microbiota is involved in the regulation of mucosal barrier function, immune balance, prevention of pathogen infection, vitamin synthesis and metabolism (Gibson, 2009).

Our results suggest that the decrease in *L. reuteri* may be related to RTX-induced mucositis. Previous studies have shown that the absence of *L. reuteri* leads to colic in infants, *L. reuteri* supplementation alleviates the damage of DSS-induced colitis in mice, and *L. reuteri* can be used to treat enteritis in IL-10-deficient mice (Madsen et al., 1999; Liu et al., 2012; Bene et al., 2017; Mu et al., 2017, 2018; Savino et al., 2018). Other studies have shown that *L. reuteri* may help to alleviate chronic inflammation (Madsen et al., 1999; Liu et al., 2012; Mu et al., 2017, 2018). All these studies have illustrated a close relationship between *L. reuteri* and inflammation.

We used antibiotics to reduce the abundance of *L. reuteri* in the intestine of mice. We found that RTX induced intestinal mucosal injury and aggravated inflammatory reactions, with marked inflammatory cell infiltration. However, our combined antibiotics were broad-spectrum antibiotics that are mainly used for anaerobic bacteria that not only remove *L. reuteri* but also reduce the content of many intestinal microbiota (Routy et al., 2018; Zhou et al., 2018; Yu et al., 2019), which does not exclude the role of these microbiota in the process of RTX-induced mucositis. Therefore, we designed an additional experimental control group to study the effect of *L. reuteri* on mucositis. The results revealed that *L. reuteri* supplementation alleviates intestinal tissue injury and the inflammatory response induced by RTX. Detection of Claudin-1 in intestinal tissue showed that the damaged intestinal mucosal barrier exhibited signs of repair. These results suggest that *L. reuteri* alleviates RTX-induced intestinal mucositis. However, the intestinal microbial community is a complex system, and its stability is closely related to the normal digestion, metabolism and immune regulation of the host. The occurrence of intestinal mucositis is not limited to the influence of a single strain but also involves the network regulation mechanism of the formation of other strains or metabolites. The relationship between other strains and RTX-induced intestinal mucositis needs further study. Previous studies have described the mechanism by which the intestinal microbiota regulates the efficacy of anticancer drugs, which can be summarized as the framework of transport, immune regulation, metabolism, enzyme degradation, and diversity reduction (Alexander et al., 2017; Roy and Trinchieri, 2017).

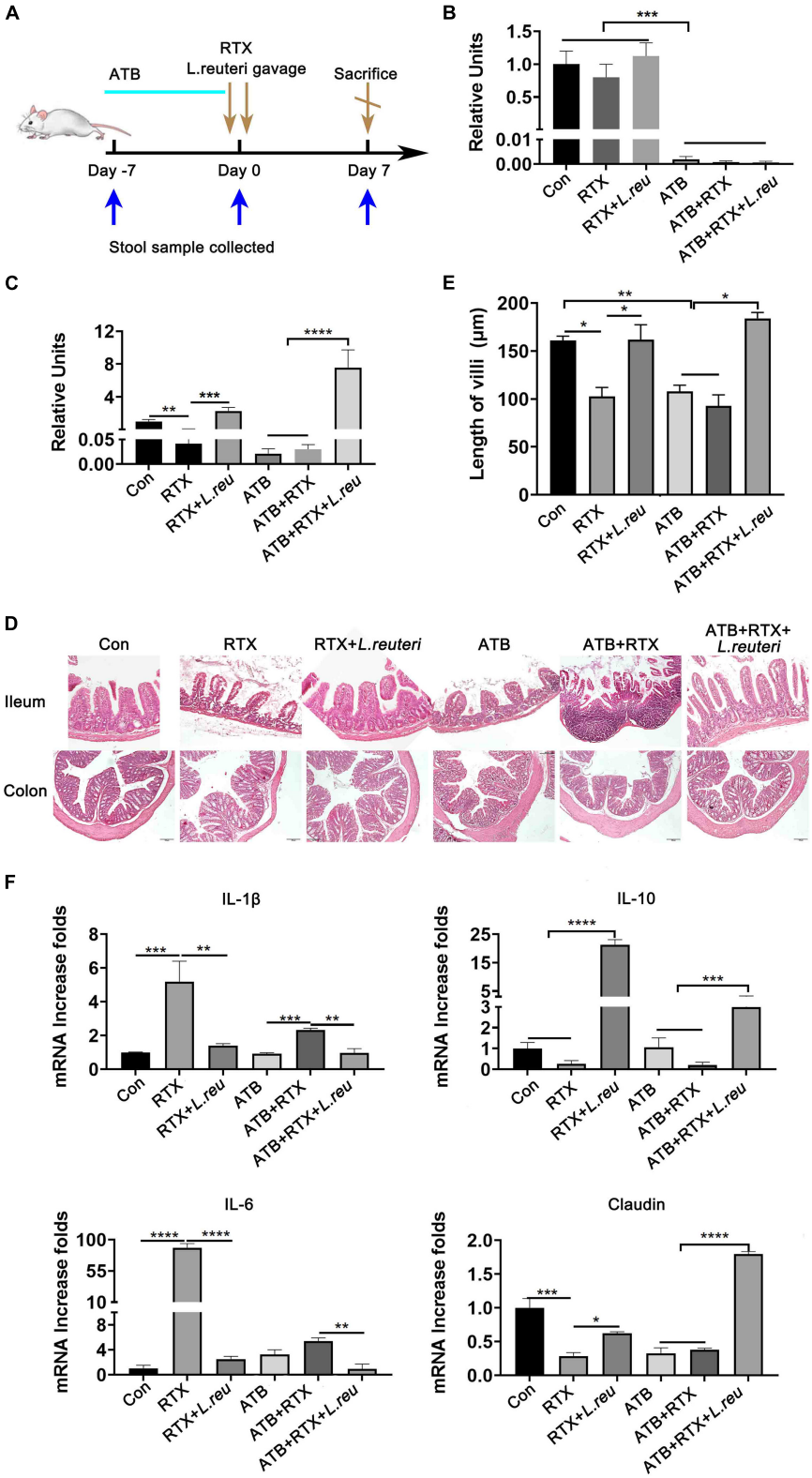

FIGURE 6 | *Lactobacillus reuteri* attenuates RTX-induced inflammatory injury. **(A)** Experimental schedule. **(B)** Relative abundance of *L. reuteri* in the intestine of mice detected by qPCR after 5 days of antibiotic administration. **(C)** Relative abundance of *L. reuteri* in the intestine detected by qPCR after 5 days of *L. reuteri* gavage. **(D)** H&E staining of the ileum and colon (magnification, 40×). **(E)** Villus length of the ileum. **(F)** mRNA expression levels of IL-1β, IL-6, IL-10, and claudin in the ileum of mice. Data are expressed as the mean ± SEM. $p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}P < 0.0001$. Con, control; ATB, antibiotic treatment; *L. reu*, *L. reuteri*.

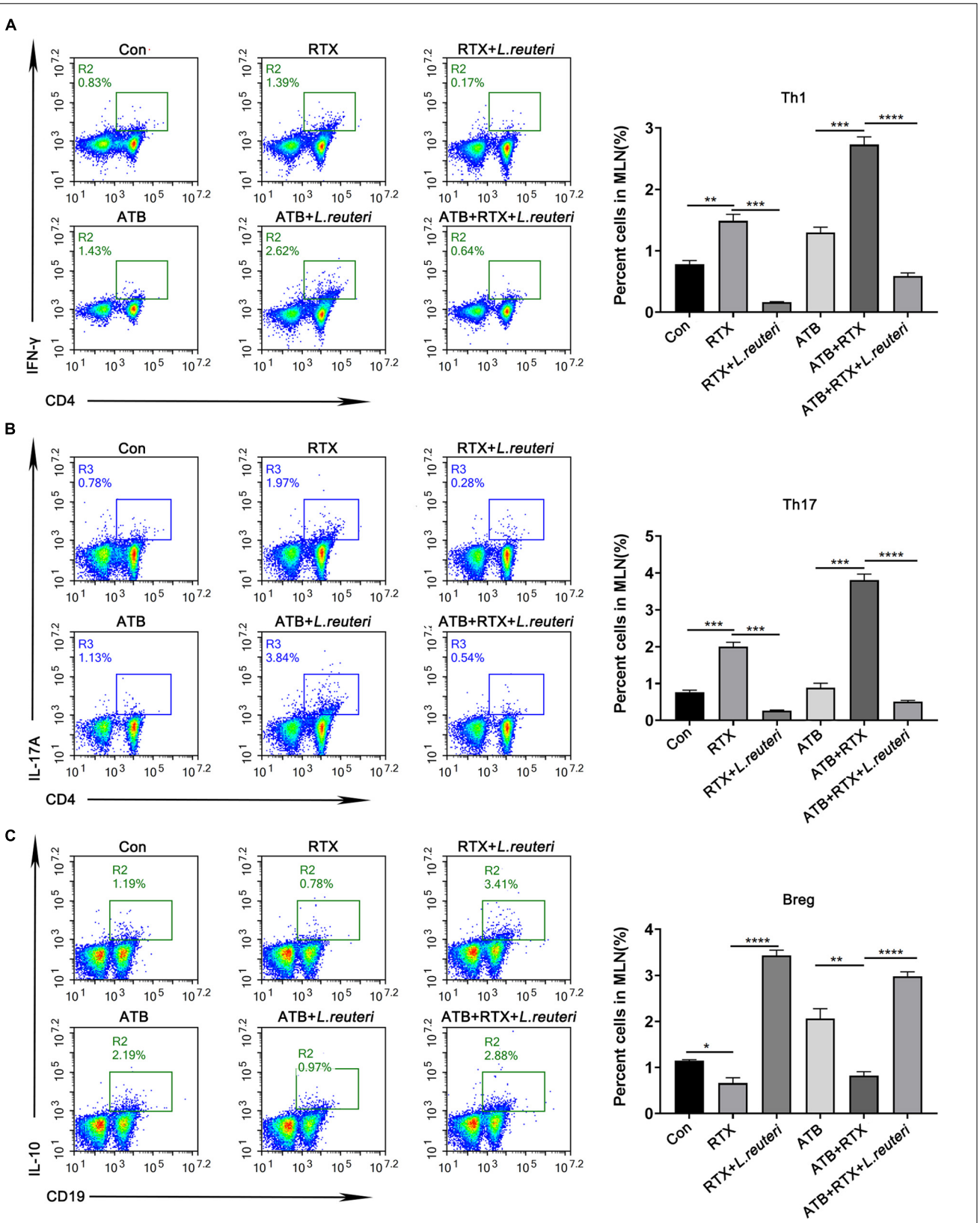

FIGURE 7 | *Lactobacillus reuteri* supplementation alleviates Th cell imbalance induced by RTX and inhibits Th1 and Th17 immune responses. **(A)** Th1 cells, **(B)** Th17 cells, and **(C)** Breg cells in MLN cells. Data are expressed as the mean ± SEM. $p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}P < 0.0001$.

A previous study reported that chemotherapy drugs lead to the destruction of intestinal structure, accompanied by the transfer of some symbiotic bacteria to the secondary lymphoid organs of mice, stimulating the response of Th1 and Th17 cells, and the antitumor effect of the drug was weakened after antibiotic treatment (Viaud et al., 2013; Yu et al., 2019). These data suggest that intestinal microbial translocation caused by intestinal mucosal damage induces an immune response and affects the efficacy of drugs. In this study, RTX caused intestinal atrophy, infiltration of inflammatory cells and destruction of the intestinal mucosal barrier. Therefore, we speculate that RTX-induced intestinal damage may lead to an imbalance in the intestinal microbiota and trigger an immune response. Results demonstrated that Th1 and Th17 cells in MLNs were significantly increased in response to RTX treatment. However, after *L. reuteri* supplementation, Th cells decreased while Breg cells increased in MLNs. Therefore, *L. reuteri* may alleviate the gastrointestinal toxicity induced by RTX through Th cells.

Regulatory B cells (Bregs) is a kind of immunosuppressive cells, which can inhibit a variety of immunopathology by producing cytokines such as interleukin-10 (IL-10). Luu et al.'s (2019) research shows that the anti-inflammatory effect of SCFA (especially pentanoate) was not only reflected in strong upregulation of IL-10 production in Bregs but also by a potent suppression of pathogenic Th17 cell phenotype. Yan et al. showed that *L. reuteri* produce short chain fatty acids (SCFAs) as a major fertilization product (Stewart et al., 2009; Den Besten et al., 2013; Furusawa et al., 2013). In addition, Nagpal et al. (2018) and others believe that the SC FA content in the intestine will increase after *L. reuteri* supplementation. It is speculated that *L. reuteri* may affect the content of Bregs by regulating the metabolism of SCFA. However, the specific mechanism still needs to be further explored.

We demonstrated that *L. reuteri* alleviates the upregulation of inflammatory cytokines in MLN cells stimulated by LPS *in vitro*. Therefore, *L. reuteri* alleviates the inflammatory stimulation caused by RTX and inhibits the local and systemic immune response caused by intestinal mucosal damage in response to RTX. Although this study shows some promising results, it also has limitations. RTX is an anticancer drug widely used in non-Hodgkin's lymphoma. It is also related to changes in the intestinal flora. We should conduct more experiments in cancer models not limited to normal mice to verify the relationship between the gut microbiota and RTX treatment. In conclusion, our data suggest that the regulation of intestinal microbiota may be a new way to reduce the toxicity of anticancer drugs.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Protection Committee of Sichuan University (Sichuan, China).

AUTHOR CONTRIBUTIONS

LY, BaZ, and BiZ contributed to conception and design of the study. BaZ organized the database. CD performed the statistical analysis. BiZ wrote the first draft of the manuscript. RZ, DX, and YT wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.645500/full#supplementary-material

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Gut–Lung Axis: Microbial Crosstalk in Pediatric Respiratory Tract Infections

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The gut microbiota is an important regulator for maintaining the organ microenvironment through effects on the gut-vital organs axis. Respiratory tract infections are one of the most widespread and harmful diseases, especially in the last 2 years. Many lines of evidence indicate that the gut microbiota and its metabolites can be considered in therapeutic strategies to effectively prevent and treat respiratory diseases. However, due to the different gut microbiota composition in children compared to adults and the dynamic development of the immature immune system, studies on the interaction between children's intestinal flora and respiratory infections are still lacking. Here, we describe the changes in the gut microbiota of children with respiratory tract infections and explain the relationship between the microbiota of children with their immune function and disease development. In addition, we will provide perspectives on the direct manipulation of intestinal microbes to prevent or treat pediatric respiratory infections.

Keywords: gut microbiota, respiratory tract infection, pediatric, immune system, gut–lung axis

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INTRODUCTION

The intestinal tract is home to about 40 trillion microbiota, and the total number of its genes is about 150 times that of human genes. Given the size of the intestinal flora, it is also known as the "second genome", "second brain", or "gut brain" of the human body. Compared with the microbiota in other parts of the human body, the intestine contains huge bacterial groups and rich species. Therefore, it influences other organs and human body functions in diverse ways. It will soon be commonplace to treat various diseases with consideration of the intestinal microbiota.

INTESTINAL FLORA INVOLVED IN SYSTEMIC IMMUNITY

The gut microbiota is an increasingly recognized component of the human body that shows dynamic changes with age, region, diet, and medication (1) (**Figure 1**). Compared with other parts of the body, the intestinal microbiota possesses enormous bacterial taxa and a rich variety of species, and they are relatively easy to be used as targets for biological intervention. The roles of intestinal microbes include stimulating pattern recognition receptors (PRRs) as bacterial antigens, facilitating the maturation of host immune system (2), regulating the secretion of protective immunoglobulin A

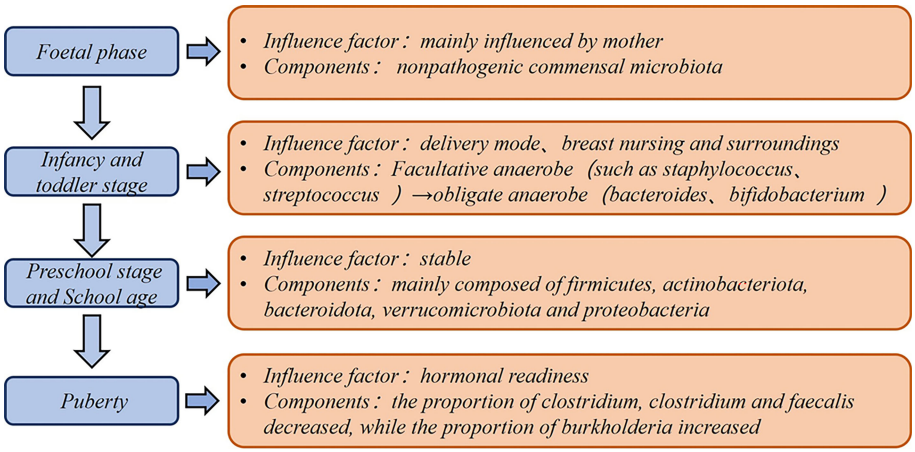

FIGURE 1 | Dynamics of intestinal flora in children.

(IgA) in the intestine (3), affecting the synthesis and transport of neurotransmitters (4), and helping organisms synthesize vitamins and bile acids (5). These events obviously have profound impacts on the host's immune response, nutrition metabolism, and cognitive function (6).

Despite the intestinal partition, the gut microbiota can promote inter-organ communication (5). This is achieved through microbe-produced metabolites. Short-chain fatty acids (SCFAs) are considered significant intestinal microbial metabolites, and acetic acid (C2), propionic acid (C3), and butyric acid (C4) account for approximately 95% of all SCFAs (7). SCFAs engage in cellular carbohydrate and fatty acid metabolism by regulating cell chromosome acetylation modification status through histone deacetylase inhibition (8, 9) or activating cells through the SCFA-G-protein receptor signal transduction pathway. These metabolites can promote immune cell maturation and maintain and regulate intestinal homeostasis *in situ*, but they also pass through intestinal tract into capillaries and transfer to major organs in the circulation. SCFAs have critical functions in the systemic action of the gut microbiota and play important roles in defense against infection, mitigation of autoimmune diseases, and anti-tumor therapy. In recent years, the description of the gut microbiota and its metabolites involved in respiratory diseases has gradually become more elaborate. With regard to the microbes themselves, microbial surface molecules like lipopolysaccharide (LPS) and lipoteichoic acids can be recognized by the immune system and induce corresponding humoral and cellular immune processes that can improve the host immune system. In controlled experiments on specific pathogen-free (SPM) mice and bacteria-free mice, SPF mice exhibited increased lymphatic tissue activity, more intestinal Peyer's patches, and greater antibody secretion with less susceptibility to systemic inflammation (10–12). The mucosa is the body's first line of defense against external pathogens.

The intestinal microbiota is essential for maturation of mucosal-associated immune tissues (MALT), which can induce the development of gut-associated immune tissues (GALT). Peyer's patches, mesenteric lymph nodes, and isolated lymphoid follicles (ILFs) are important secondary lymphoid tissues of GALT and vital sites for the B cells that produce IgA to neutralize pathogenic microorganisms (13, 14). These structures are crucial habitats for IgA-producing plasma cells. In terms of their metabolites, SCFAs could ease microbial-induced allergic lung inflammation by acting on Th2 cells, while antibiotics such as vancomycin increase the incidence of allergic lung diseases by reducing intestinal SCFA levels (15). Moreover, SCFAs have the ability to alleviate pulmonary fibrosis and make patients less susceptible to further infection (16). C2–C4 can inhibit neutrophil cytotoxicity (17), and C3 has been reported to inhibit macrophage function in the intestine (18) by inducing the apoptosis of inflammatory dendritic cells (19), thereby affecting immunity in the whole body and all major organs. SCFA affects cells in various ways in different microenvironments. Various organs and microenvironments can induce the differentiation of initial T cells into effector T cells or T regulatory cells (9, 20, 21). Regarding the effect of SCFAs on B cells, one report indicated that it is associated with shaping intestinal homeostasis and maintaining IgA expression levels in the bronchial-associated mucosal immune system (22). Other recent findings suggest that SCFAs can protect against arthritis by skewing regulatory B cell differentiation (23).

DYNAMIC DEVELOPMENT OF THE PEDIATRIC INTESTINAL FLORA AND IMMUNE SYSTEM

Although the fetus was previously considered to exist in a sterile uterine environment, the results of a 16S ribosomal DNA-based and whole-genome shotgun metagenomic study based of 320 subjects show that the placenta also has unique microbial niche

that consists of nonpathogenic commensal microbiota (24). A recent study found that a small number of premature babies had placentas containing bacteria of the same origin as their mothers' mouths (25). This suggests that even at the embryonic stage, the fetus is already in contact and exchanging information with microorganisms. In a retrospective analysis of newborn feces, an upward trend appeared in the variety and amount of intestinal microbes (26). The infant gut microbiota shows a rapid increase until 1 year of age. From 1 to 5 years of age, the growth rate of flora diversity decreases and the composition becomes more stable, but their gut microbiota is lower in both number and species compared to adults (27). HIT Chip microarray analysis indicated that the most striking differences between young children and adults are found in *Actinobacteria*, *Bacilli*, *Clostridium cluster IV*, and *Bacteroidetes* phylum-like groups (28). In terms of dietary habits including fat, protein, sugar, and fiber intake, nutritional intake in infants and older children can lead to dramatic differences in the colonized taxa (29). Before children reach puberty (7–12 years old), their gut microbiota exhibit different functions including vitamin synthesis, amino acid degradation, and oxidative phosphorylation (30).

Compared with adults, the shaping of the pediatric immune system is more dependent on education from the external environment. The gut microbiota provides a window into the immune maturation of newborns. After weaning from breast milk, the intestinal microflora expands and produces related SCFAs, giving the baby a mucosal immune stimulation and promoting immune system maturation (31, 32). Bacteria are necessary for ILF development. The addition of solid foods about 2 weeks after weaning can increase bacteria colonization and is thus conducive to enhancing infant gut microbiota integrity (13).

Some hypotheses suggest that children's immune systems are not imperfect, or rather, offer them better protection at an early age. Compared to adults, children's immune systems tend to be more protective to external invasions, but they also generate the corresponding immune response once activated (33). The pediatric immune system is in the process of development, from simple to complex and from tolerant to sensitive. Early adaptive immunity is not well developed, and relies more on the PRRs of innate immunity to recognize danger-related molecular patterns or pathogen-related molecular patterns (34, 35). Neonates have fewer neutrophils than adults, and the high level of interleukin (IL)-6 in infants will diminish neutrophil recruitment and thus inflammation (36, 37). Natural killer (NK) cells play a prominent role in controlling viral infections, but studies on human fetuses have shown that NK cells are extremely sensitive to transforming growth factor-β inhibition, thereby reducing cytotoxicity and interferon-γ production (38, 39). As for cellular and humoral immunity, infant T cell immunity skews towards Th2 cells, with reduced Th1 cell differentiation and immune responses (40). Due to less antigenic stimulation, neonates have fewer memory T cells and memory B cells than adults (41), and therefore are prone to mild responses following secondary stimulation. In a study of human volunteers of different ages (42), the proportions of CD4 RTE cells, transitional B cells, and CD8 RTE cells decreased with age; furthermore, the vitality of the thymus and bone marrow decreases. Several inflammatory cell subsets including Th1 cells, CD4IL-2 cells, CD8IL-2 cells, and invariant NK T cells are up-regulated with age. In contrast, Th2 and Th17 cells did not show age-related changes.

CROSSTALK BETWEEN GUT MICROBIOTA AND CHILDHOOD RESPIRATORY INFECTIONS

In adult studies, the gut microbiota can directly regulate the immune function of the lungs (43). In an experiment to detect specific antibodies after influenza virus infection, neomycin-sensitive bacteria were shown to be related to the production of immune responses in the lungs (44). Dysbiosis of intestinal microbes can induce mice to have different Th cell responses to influenza virus infection. Th1 and Th2 cells are more controlled by the gut microbiota, with a weaker effect on Th17 cells (45). Follicular helper T cells are deficient in germ-free mice, leading to a lack of B cell humoral immunity and impaired IgA^+ plasma cell function, which impacts the development and severity of colitis (46). Moreover, antibiotic use can dysregulate the intestinal microbe composition, which triggers the overgrowth of yeasts and more severe pulmonary allergic reactions (47). Common respiratory tract infections in children have been associated with the gut microbiota to some extent (48, 49). First of all, children are exposed to the outside world while their microbial colony gradually grows, and the immune system is constantly exposed to foreign antigens, which guide continuous immune system improvement. The gut microbiota shapes the immune system in children, providing constant reserves for the infectious diseases (50).

According to the results of 16S rRNA gene sequencing, *Lachnospira*, *Veillonella*, *Faecalibacterium*, and *Rothia* were reduced in the intestines of children with asthma, and fecal levels of SCFAs and intestinal-liver metabolites were also disordered. These four bacteria are associated with disease progression, and researchers proposed that low abundance of these bacteria contributes to a higher risk of developing asthma before the age of 3 (51). Respiratory syncytial virus (RSV) often causes severe lower respiratory tract infections in infants, but its pathogenesis remains obscure. Some studies have shown that the infant intestinal microbe niche is significantly related to RSV infection severity. The 16S rRNA gene sequencing results of the feces of infants infected with RSV showed higher abundance of S247, *Clostridiales*, *Odoribacteraceae*, *Lactobacillaceae*, and *Actinomyces* in moderately and severely infected patients compared to normal infants. The *Moraxellaceae* flora decreases in children with severe RSV (52). The correlation between the intestinal microbe niche and respiratory diseases in infants and young children is gradually being revealed for variety of diseases. A study of infant bronchitis (53) revealed that the four dominant bacterial groups in the intestinal tract of normal healthy infants were: *Escherichia*-dominant profile (30%), *Bifidobacterium*-dominant profile (21%), *Enterobacter/Veillonella*-dominant

profile (22%), and *Bacteroides*-dominant profile (28%). However, the predominant taxa in children with bronchitis changed to: *Enterobacter/Veillonella*-dominant profile (15%), and *Bacteroides*-dominant profile (44%). Researchers hypothesized that the *Bacteroides*-dominant profile may put infants at a higher risk of developing bronchitis. This lays a theoretical foundation for modifying the gut microbiota to treat or prevent bronchitis. Lei Li and colleagues (54) reported reduced gut microbiota diversity in pediatric patients with recurrent respiratory tract infections. Conversely, patients were significantly enriched in *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia*, *Tenericutes* phylas and *Enterococcus*, *Faecalibacterium*, *Bifidobacterium*, and *Eubacterium* generas. However, *Eubacterium*, *Faecalibacterium*, and *Bifidobacterium* decreased. Compared to their healthy peers, children with pulmonary tuberculosis had reduced intestinal microbe diversity with enrichment of the pro-inflammatory bacteria Prevotella and the opportunistic pathogen *Enterococcus*, and decreases in the probiotics *Ruminococcaceae*, *Bifidobacteriaceae*, and *Faecalibacterium prausnitzii* (55). It can be seen that there is a close relationship between intestinal flora and respiratory tract infection,different respiratory tract infection pathogens can cause different changes in intestinal flora (**Table 1**) .

The direction of the interaction between the gut microbiota and respiratory tract infections remains unclear. Whether fluctuations in the gut microbiota caused by environment, diet, or genetic factors increase the risk of respiratory tract infections, or whether respiratory tract infections skew the gut microbiota requires further investigation. What is certain is that intestinal microbial communities can indeed shape the pediatric immune system. In clinical treatment, altering the intestinal bacteria can indeed treat and prevent severe symptoms of respiratory diseases (60).

After cessation of breastfeeding, the intestinal microflora will expand and produce SCFAs, which will give the infant mucosal immune stimulation and promote immune system maturation (61). Some theories suggest that compared with adults, the pediatric immune landscape is indeed biased towards tolerance. For example, adults develop severe clinical symptoms of SARS-CoV-2 (COVID-19) infection, but only very few infected children develop dramatic upper respiratory symptoms (62). Children and adolescents often present with mild or no COVID-19 symptoms. In addition, young children have a very high probability of developing viral respiratory infections but with very low disease symptoms (63). This may be due to the fact that children are constantly undergoing gut microbiota remodeling and have a higher immune tolerance, which helps them resist the devastating effects of cytokine storms on the body.

GUT MICROBIOTA-BASED TREATMENT FOR RESPIRATORY INFECTIONS IN CHILDREN

The gut microbiota provides protection against respiratory diseases by shaping the immune system. This role is even more pronounced in diseases for which innate immunity is involved in the early stage (64). For example, commensal bacteria transfer therapy can induce the production of neonatal intestinal innate lymphoid cells (ILCs), thereby boosting plasma granulocyte colony-stimulating factor levels and neutrophil numbers and improving IL-17-dependent sepsis tolerance (65). Resistance to respiratory infections can be significantly enhanced by using microbiome-related treatments rather than antibiotic therapies. LPS supplementation can increase the expression of IL-6 and IL-1, as well as the immune response to *E. coli*-induced pneumonia *via* Toll-like receptor 4 signaling (66). Germ-free mice secreted superfluous IL-10, creating an immunosuppressive microenvironment, which made them more susceptible to bacterial infection. Pretreatment with LPS can decrease IL-10 production and increase the infiltration of neutrophils into infected lungs (67). Intestinal segmented filamentous bacteria are critical in the formation of Th17 immunity against *S. aureus* pneumonia (68). The gut microbiota facilitates the maturation of IL-22^{+}ILC3, offering host resistance to bacterial pneumonia (69). Microbial-based therapies are widely used in clinical trials in the form of probiotics. Compared with conventional antibiotics, intestinal microbiota transplantation can achieve better therapeutic outcomes (70, 71). Probiotics and supplements for infants and young children can alter the intestinal microbe composition (72), thereby reducing the risk of respiratory virus infection in premature infants. Supplementing *Lachnospira*, *Veillonella*, *Faecalibacterium*, and *Rothia* to mice with pneumonia significantly alleviated the symptoms, demonstrating the protective effect and therapeutic potential of

TABLE 1 | Changes in intestinal flora caused by respiratory pathogen infection.

Respiratory pathogens	Changes in intestinal flora	References
Respiratory syncytial virus	Firmicutes↓, S247↑, Clostridiales↑, Odoribacteraceae↑, Lactobacillaceae↑, Actinomyces↑	(52)
Influenza virus	Enterobacter↑, Akkermansia↓, Desulfovibrio↓, Lactobacillus↓	(56)
Mycoplasma	Bifidobacterium↓, Lactobacillus↓, Colibacillus↑	(57)
Streptococcus pneumoniae	Lactobacillus↓, Bifidobacterium↓, Bacteroidetes↓, Colibacillus↑	(58)
Staphylococcus aureus	Total aerobic↑, Enterococcus↑, Total anaerobic↓, *Clostridium perfringens*↑	(59)
Mycobacterium tuberculosis	Pro-inflammatory bacteria Prevotella↑, Opportunistic pathogen Enterococcus↑, Probiotics Ruminococcaceae↓, Bifidobacteriaceae↓, *Faecalibacterium prausnitzii*↓	(55)

specific gut microbiota taxa (73). The combination of vitamin C and probiotics (*Lactobacillus acidophilus CUL21*, *Lactobacillus acidophilus CUL60*, *Bifidobacterium bifidum CUL20*, and *Bifidobacterium animalis* subsp. lactis CUL34) can effectively lower the infection rate of the upper respiratory tract (74).

FUTURE PERSPECTIVES

The gut–lung axis theory is gradually being advanced and accepted. Stable development of the intestinal microbiota in children can improve resistance to pathogens that cause respiratory tract infections. As the organ with the largest microbial ecosystem, a healthy intestinal niche often shows rich diversity. During different respiratory diseases, microbes and products in the intestinal tract will turn into morbid types with harmful traits. In the disease state, there is more communication between the intestine and lungs. Mucosa, secretions, pH, and other changes act on target cells and become messengers between the gut and other organs.

In children, the immune system and gut microbiota are undergoing vigorous development. Given that the pediatric gut microbiota is undergoing an integrated developmental process, it is important for us to consider the potential impact of prematurely introducing or eliminating a certain bacteria. Shifts in gut microbiota early in life or before weaning may provoke an increase in asthma-related immune responses (49). Mild dietary interventions also make sense to treat respiratory infections in children. Given the development of the gut microbiota taxa, we should probably consider using antibiotics with caution. Microbiological intervention strategies should also be considered for children with respiratory infections with marked changes in the gut microbiota due to obesity, complicated enteritis, or immunodeficiency.

AUTHOR CONTRIBUTIONS

WZ was in charge of literature retrieval and paper writing. YW, HL, and CJ were in charge of literature retrieval. LH was in charge of reviewing the papers. All authors contributed to the article and approved the submitted version.

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