THE ROLE OF THE GUT MICROBIOTA IN HEALTH AND INFLAMMATORY DISEASES

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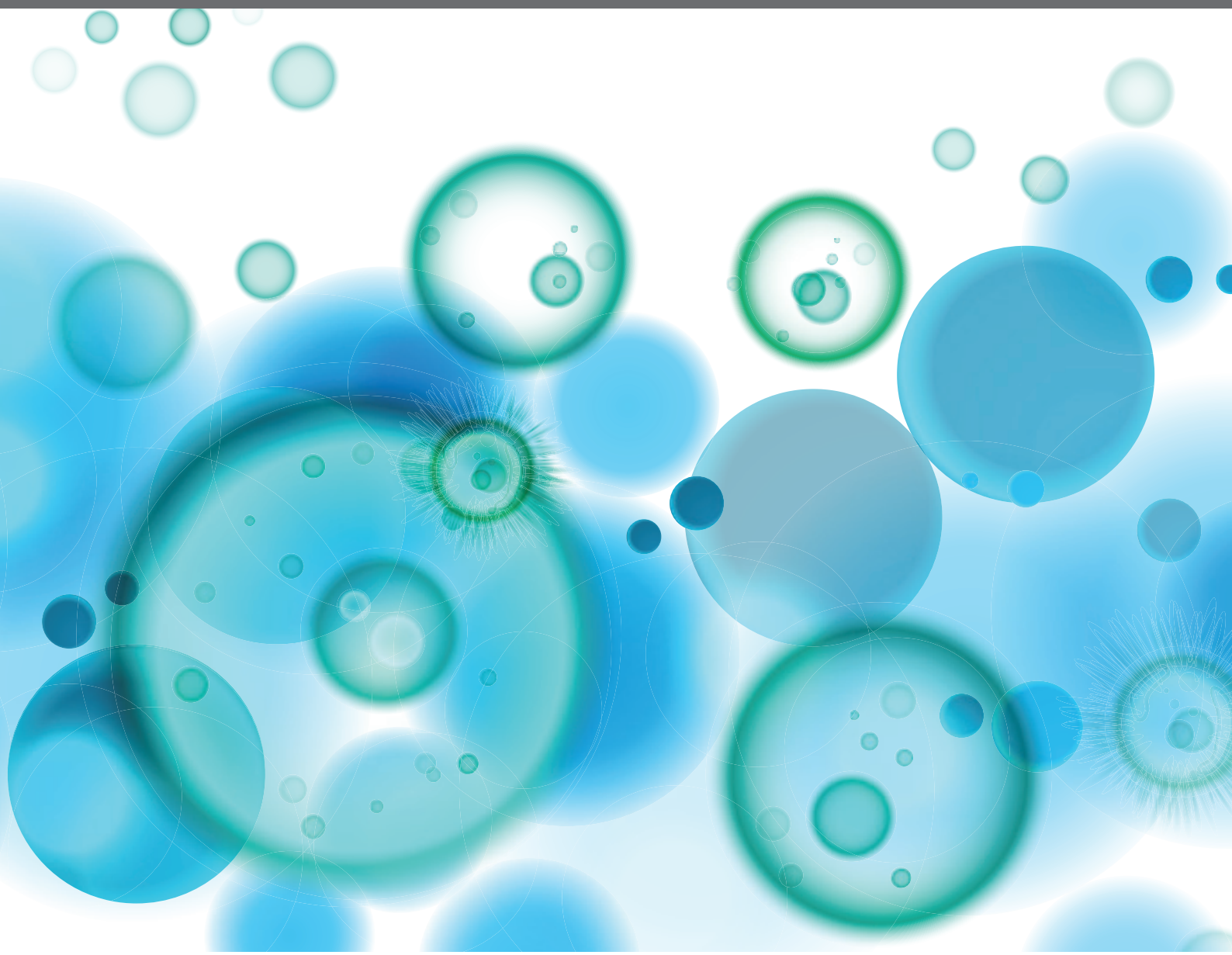

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THE ROLE OF THE GUT MICROBIOTA IN HEALTH AND INFLAMMATORY DISEASES

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EDITORIAL
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Editorial: The Role of the Gut Microbiota in Health and Inflammatory Diseases

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Keywords: microbiota, immunomodulation, gut - associated lymphoid tissues (GALT), inflammation, autoimmunity, microbiome

Editorial on the Research Topic

The Role of the Gut Microbiota in Health and Inflammatory Diseases

Soon after birth, humans are colonized with a vast number of microbes, collectively termed the microbiota. Among the microbiota, commensal bacteria help shape and regulate a number of the host's physiological processes, including the immune system. Alterations in the gut microbial community (dysbiosis) have been linked with multiple diseases. Therefore, the identification of commensal bacteria that are depleted or enriched in the context of pathological conditions, and the mechanisms by which they predispose or protect from disease is an active area of research. In this collection, several articles discuss the biological factors and processes that are regulated by gut microbes, and the potential use of commensal bacteria as therapeutic agents to treat disease.

The diet of an individual has emerged as the strongest factor that influences the composition and function of the gut microbiota (1). The relevance of dietary factors in modulating the microbiota to maintain the immune response is highlighted by current evidence linking gut dysbiosis with multiple diseases (Yap and Marino). Although the contributions of commensal bacteria to T and B cell function have been studied extensively, its effect on intraepithelial lymphocytes (IELs) is poorly defined. Yap and Mariño discuss the current knowledge on the regulation of IELs by gut bacteria whereas Chen et al. use of antibiotic treatment and germ-free (GF) mice to illustrate the importance of commensal bacteria in regulating the function of IELs against gut microbes. In this study, the authors showed that in the absence of microbes, there is a significant reduction of $CD8ab^+$ IELs, which impacts their ability to produce anti-microbial peptides (Chen et al.). Although the data are currently limited, there is evidence to suggest that the gut microbiota influences the function of IELs.

In a healthy individual, immune-tolerance is maintained by balancing levels of anti-inflammatory $CD4^+FoxP3^+$ regulatory T cells (Tregs) and proinflammatory Th17 cells. Certain commensals such as segmented filamentous bacteria (SFB) can induce Th17 cells (2), whereas others such as *Clostridium* species (3), *Bacteroides fragilis* (4), and *Prevotella histicola* (5) induce Tregs. Dysbiosis due to either reduced Treg induction and/or increased Th17 induction, can promote proinflammatory conditions, which can predispose individuals to, or exacerbate, disease (6). The implications of generating immunotolerance by inducing tolerogenic intestinal antigen-presenting cells, Tregs, and immunoregulatory cytokines have been discussed (Vitetta et al.). In this special issue, two studies utilized a model of subacute ileitis

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to decipher the role of the human microbiome in intestinal inflammation, and in extra-intestinal and systemic inflammation (Bereswill et al.; Heimesaat et al.). Both studies utilized a human microbiota-associated (hma) mouse model, in which abiotic mice were colonized with human feces and orally infected with a low-dose of *Toxoplasma gondii* to induce subacute ileitis. Using this model, Heimesaat et al. show the relevance of ileum acquired multidrug-resistant (MDR) *Pseudomonas aeruginosa* in causing systemic inflammation. Bereswill et al. utilize the hma model to show the therapeutic ability of the neuropeptide, pituitary adenylate cyclase-activating polypeptide, to treat subacute ileitis. These two studies highlight the relevance of using this unique model system to understand the pathogenesis of intestinal and systemic inflammatory diseases and its usefulness in testing novel therapies.

As the colon harbors the maximum density of commensal bacteria, the association of gut dysbiosis with colon pathology is not surprising. Okamoto et al. reported a patient with Cap polyposis (a rare heredity disease of the colon) successfully treated with antibiotics. The treatment with antibiotics caused the improvement in disease, that was associated with a significant change in the composition of the gut microbiota. Thus, the enrichment of pathobionts that are associated with gut dysbiosis is an emerging common theme among intestinal and extra-intestinal/systemic diseases. Restoring the microbial composition to a healthy state, either directly using gut commensal bacteria as therapeutic agents, or indirectly using chemical drugs (e.g., antibiotics), can significantly alleviate symptoms associated with these diseases.

Several manuscripts published previously and in this special issue report the use of gut commensal bacteria (single-strain or probiotic mixture) as a therapeutic option to correct dysbiosis and thereby treat disease (Maddaloni et al.; Shahi et al.; Swartwout and Luo; Vitetta et al.) As gut dysbiosis shifts the balance toward inflammatory, as opposed to immunoregulatory cell subsets, treatment with commensal bacteria that induce immunomodulation can restore homeostasis. Shahi et al. describe in their work that the human commensal bacteria, *Prevotella histicola,* is as effective as the MS drug Copaxone in suppressing disease in experimental autoimmune encephalomyelitis (EAE), an animal model of MS. Of note, the use of *Prevotella* is of particular relevance to human MS as it is decreased in abundance in untreated MS patients (7, 8). Correspondingly, the treatment of MS with disease-modifying therapies restores levels of *Prevotella* (9).

Maddaloni et al. describe the use of a genetically-engineered *Lactococcus lactis* designed to provide protection from autoimmune diseases such as arthritis by producing enhanced levels of IL-35. Using a murine model of collagen-induced arthritis, the authors show that oral treatment with IL-35-producing *L. lactis* induced protective responses by promoting the generation of IL-10-producing Tregs and decreasing interferon (IFN)-gamma and IL-17-mediated inflammation. Zhang et al. report that the human commensal *Bacteroides fragilis* protects from antibiotic-associated diarrhea by restoring the gut microbiota's composition to a healthy state. In addition to these studies, a *B. fragilis* strain capable of expressing the capsular polysaccharide A (PSA), has been shown to possess therapeutic potential against experimental models of ulcerative colitis (10), CNS demyelinating disease (11), asthma (12), and autism spectrum disorders (13). Thus, these studies highlight the therapeutic potential of both un-manipulated (*P. histicola* and *B. fragilis*) as well as engineered human commensals (*L lactis)* to treat various intestinal (antibiotic-induced diarrhea) and extra-intestinal diseases (MS and RA).

The administration of probiotics, prebiotics, or phages can provide health benefits by promoting a balanced immunological response. This microbial-based treatment may be especially relevant during pregnancy, as pregnancy significantly alters the immune system. The concept of maternal probiotics as a mechanism to modify the maternal-infant interface and promote immune homeostasis is attractive. Swartwout and Luo highlight the effects of pregnancy on the composition of the intestinal bacteria in the context of a broader discussion on the current lack of data regarding maternal probiotics and their effects on the incidence and progression of autoimmune diseases. Yet the excitement for this is limited, as additional experiments that test the approach's safety and efficacy are required.

The role of the microbiota in the generation of food allergies is an area of active research (14). One hypothesis is that food allergies are triggered by tolerance breakdown at the intestinal level (Berni Canani et al.), suggesting that understanding the role of the microbiota in breaking tolerance to diet could provide novel avenues to control food allergies. Pascal et al. propose that dysbiosis is a factor that triggers allergies, not only in the context of the intestinal milieu and food allergies but also in the skin and respiratory tract. The authors discuss the potential cellular mechanisms by which alterations of the microbiota could lead to tolerance breakdown, such as modulation of the IgE–basophil axis, alterations in the MyD88 pathway in B lymphocytes and dysregulation in the balance between Th2/Th17 cells.

Advances in sequencing techniques and computation tools have moved forward our understanding of the microbiome's complexities. These advances, discussed by Malla et al., will help catalog the gut microbial community, allowing us to harness its potential use as future diagnostic and therapeutic tools. In summary, the collection of articles in this special issue tackles some of the crucial aspects of gut microbiota in health and disease and highlights an increasing appreciation for the microbiome's contribution to human health.

AUTHOR CONTRIBUTIONS

AM and JO-R contributed equally to the organization, writing, and editing of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: AM is one of the inventors of the use of Prevotella histicola for treatment of autoimmune disease, used in this study and the patent is owned by Mayo Clinic Rochester, USA. The technology has been licensed by Mayo Clinic to Evelo Biosciences. AM received royalties from Mayo Clinic (paid by Evelo Biosciences). JO-R serves as a consultant for Symbiotics Biotherapies.

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CASE REPORT
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Dysbiosis-Associated Polyposis of the Colon—Cap Polyposis

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Cap polyposis is a rare gastrointestinal disease characterized by multiple inflammatory polyps located between the distal colon and the rectum. Despite the lack of clarity regarding its pathogenesis, mucosal prolapse, chronic inflammatory responses, and *Helicobacter pylori* infection are considered key contributors to the development of this disease entity. Although it is now generally accepted that dysbiosis of gut microbiota is associated with intestinal and extra-intestinal diseases, alterations of intestinal microbiota have been poorly defined in cap polyposis. Here, we report a patient with *H. pylori*-negative cap polyposis who was successfully treated with antibiotics and exhibited dramatic alterations in intestinal microbiota composition after antibiotic treatment. The patient was treated with oral administration of ampicillin and metronidazole and showed regression of cap polyposis 6 months after antibiotic treatment. Fecal microbiota analysis using the next-generation sequencing technology revealed a significant alteration in the intestinal microbiota composition following antibiotic treatment—a marked reduction of *Blautia*, *Dorea*, and *Sutterella* was observed concomitant with a marked increase in *Fusobacterium*. These data suggest that cap polyposis may originate from dysbiosis and that microbiome-targeted therapy may be useful in this disorder.

Keywords: cap polyposis, intestinal microbiota, next-generation sequencing, inflammation, antibiotics

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HIGHLIGHTS

- Cap polyposis patient.
- Cap polyposis is considered as multiple inflammatory polyps.
- Regression of colonic polyposis after antibiotic treatment.
- Next-generation sequencing reveals dynamic changes in the intestinal microbiota composition following antibiotic treatment.
- Identification of pathogenic bacteria associated with cap polyposis.

INTRODUCTION

Cap polyposis is a rare disease characterized by multiple inflammatory polyps that are covered by a cap of fibrinopurulent mucus and are located between the distal colon and the rectum (1–3). Patients

Abbreviations: IBD, inflammatory bowel disease; MPS, mucosal prolapse syndrome; NGS, next-generation sequencing; OTU, operational taxonomic unit; rRNA, ribosomal RNA.

usually present with abdominal pain, blood and/or mucus in diarrheal stool, and hypoproteinemia (1–3). Microscopically, the colonic polyps are characterized by a cap of fibrinopurulent exudates, distorted glands, fibromuscular obliteration of the lamina propria with inflammatory cell infiltration (1–3). Although the pathogenesis of cap polyposis remains unknown, the clinical and histopathological features of this disorder resemble those observed in patients with mucosal prolapse syndrome (MPS) (4). Therefore, mucosal prolapse secondary to impaired colonic motility has been considered a possible etiological contributor to cap polyposis (4). Several reports have shown regression of cap polyposis following the use of infliximab (2) or steroid (3), thereby demonstrating the possible involvement of chronic inflammatory responses in the development of cap polyposis. Reportedly, eradication of *Helicobacter pylori* is effective in the management of cap polyposis (5, 6). However, *H. pylori* were not detected in the colonic mucosa in any of these cases. Therefore, it is possible that unidentified intestinal bacteria, sensitive to *H. pylori*-eradication therapy, may contribute to the development of cap polyposis. Considering that eradication of *H. pylori* causes a significant alteration in the intestinal microbiota composition, these case reports suggest that dysbiosis-related immune responses may underlie the pathogenesis of cap polyposis. However, the intestinal microbiota composition has not been determined in this condition.

We report a patient with *H. pylori*-negative cap polyposis who was successfully treated using antibiotics. Fecal microbiota analysis using the next-generation sequencing (NGS) technology revealed a significant alteration in the intestinal microbiota composition pre- and post-antibiotic treatment. The results of our study strongly support the contributory role of dysbiosis in the pathogenesis of cap polyposis.

CASE REPORT

An asymptomatic 45-year-old man without a relevant past or family history of gastrointestinal disease underwent a colonoscopic and esophagogastroduodenoscopic examination for the evaluation of a positive fecal occult blood test. Colonoscopic examination revealed multiple sessile polyps in the descending colon, which showed a reddish surface covered by white mucus (**Figure 1A**).Esophagogastroduodenoscopic examination revealed multiple fundic gland polyps. Serum anti-*H. pylori* antibody titers were below the detection limit, and serum total protein and albumin levels were within the reference range, as was the complete blood cell count. Endoscopic mucosal resection was performed to determine a histopathological diagnosis of the colonic polyps. The resected specimen showed mucus-containing distorted glands and significant inflammatory cell infiltration with fibrosis in the lamina propria (**Figure 1B**) and their surface was covered by inflammatory granulation tissue and fibrinopurulent exudate. These endoscopic and histopathological findings were consistent with those typically observed in patients with cap polyposis (1–3). Thus, this patient was diagnosed with cap polyposis without *H. pylori* infection.

Helicobacter pylori infection has been considered a possible etiological contributor to the development of cap polyposis

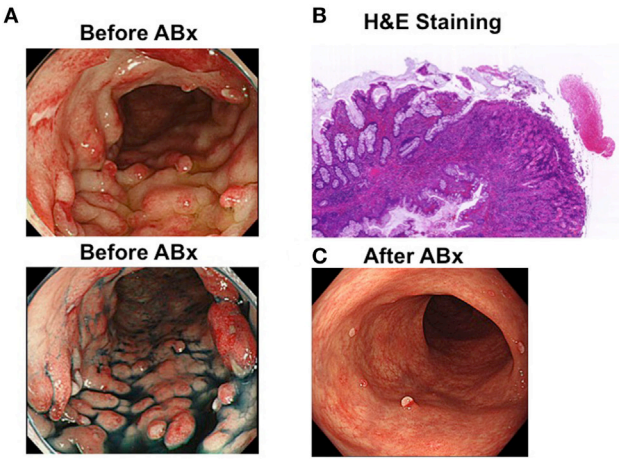

FIGURE 1 | Endoscopic and pathological findings in a patient with cap polyposis. **(A)** Endoscopic images in a patient with cap polyposis before antibiotic treatment (before ABx). Multiple sessile polyps are observed in the descending colon prior to the initiation of antibiotic treatment. Top and bottom panels show white light endoscopy and chromoendoscopy images, respectively. The polyps appear reddish in color and are covered by white mucus. **(B)** Microscopic pictures of a patient with cap polyposis. Endoscopic mucosal resection was performed to obtain a histopathological diagnosis. Low magnification revealed inflammatory polyps covered with granulation tissue and fibrinopurulent exudate. Distorted glands were also seen in lamina propria. Hematoxylin and eosin (H&E) staining. Magnification ×40. **(C)** Endoscopic images obtained from a patient with cap polyposis treated with antibiotics (after ABx). Most of the inflammatory polyps are observed to have disappeared 6 months post-antibiotic treatment.

because eradication of this organism is observed to cause regression of colonic polyps in some patients (5, 6). However, notably, *H. pylori* have not been detected in the colonic mucosa in any patient diagnosed with cap polyposis. Thus, gut bacteria sensitive to the antibiotic component of *H. pylori*-eradication therapy are likely to play a pathogenic role in patients with cap polyposis. Based on this hypothesis, this patient was treated with oral administration of ampicillin (1,500 mg/day) and metronidazole (500 mg/day) for 1 week, and regression of cap polyposis was observed 6 months post-antibiotic treatment (**Figure 1C**). This clinical course strongly suggests that antibiotic-induced eradication of pathogenic gut bacteria responsible for the development of inflammatory polyps can cause regression of cap polyposis.

FECAL MICROBIOTA ANALYSIS

Stool samples pre- and post-antibiotic treatment were subjected to fecal microbiota analysis, which was performed as previously described (7, 8) to assess any alterations in the intestinal microbiota composition. Ethical approval for this study was granted by a Review Board of the Kindai University Faculty of Medicine. DNA samples extracted from the stool were subjected to polymerase chain reaction for the amplification of the 16S ribosomal RNA (16S rRNA) V3 and V4 regions. Primer sequences are available in our previous report (8). We performed 16S rRNA sequencing using the MiSeq system (Illumina) (7, 8). Trimmomatic, Cutadapt, and Fastq-join programs were used

for sequence data processing, and operational taxonomic units (OTUs) were defined using the QIIME program (7, 8). The defined OTUs were subjected to population analysis to identify the bacterial phylum, class, order, family, and genus.

We detected 1,077 OTUs from 3 stool samples obtained pre- and post-antibiotic treatment. No major taxonomic alterations in the microbial communities were observed at the level of the phylum, order, or family (data not shown). Significant changes in the composition of fecal microbiota were noted at the genus level pre- and post-antibiotic treatment (**Figure 2A**). *Blautia* and *Dorea*, which showed a high relative abundance in the feces pre-antibiotic treatment, disappeared 1 week and 6 months post-antibiotic treatment (**Figure 2B**), and *Sutterella* disappeared 6 months post-antibiotic treatment. By contrast, the relative abundance of *Fusobacterium* was observed to have increased post-antibiotic treatment. The relative abundance of *Bifidobacterium*, *Bacteroides*, or *Veillonella* remained largely unchanged pre- and post-antibiotic treatment. This microbiota analysis suggests that regression of cap polyposis following antibiotic treatment is accompanied by a marked decrease in *Blautia*, *Dorea*, and *Sutterella* and a marked increase in *Fusobacterium*, indicating that cap polyposis might have originated from dysbiosis in this patient.

DISCUSSION

Recent progress in NGS technology has highlighted the role of an altered intestinal microbiome (dysbiosis) in human diseases (9). Inflammatory bowel disease (IBD) is a prototypical dysbiosis-related disorder mediated by abnormal immune responses to altered intestinal microbiota (9). In this study, we report a patient with cap polyposis in whom antibiotic treatment resulted in regression of multiple inflammatory polyps. Interestingly, regression of cap polyposis was associated with significant alterations in the composition of the intestinal microbiota. Thus, we propose that cap polyposis might be a dysbiosis-related intestinal disorder. It should be noted, however, that we cannot exclude two other possibilities in the regression of cap polyposis. First, spontaneous regression might have occurred in antibiotic

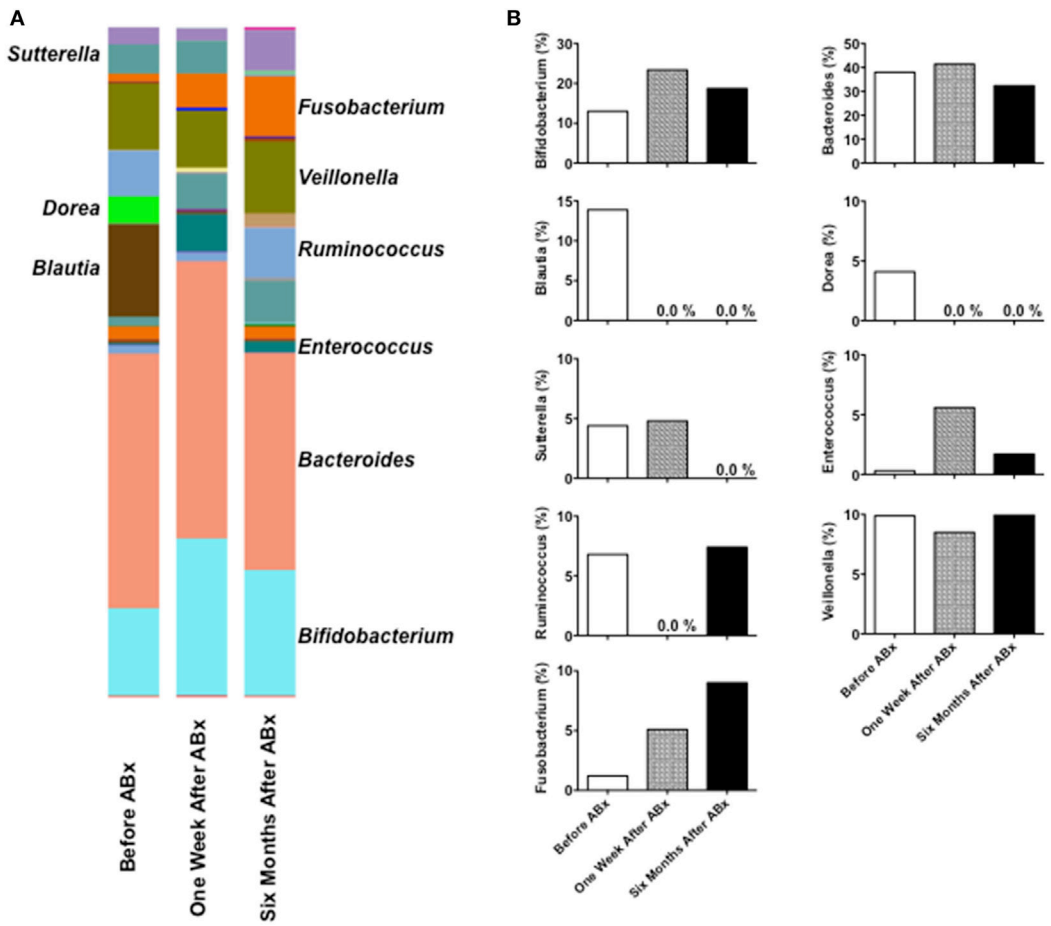

FIGURE 2 | Fecal microbiota analysis in a patient with cap polyposis. **(A)** Stool samples were obtained from a patient with cap polyposis prior to, 1 week and 6 months post-antibiotic treatment. DNA samples extracted from the stool specimens were subjected to polymerase chain reaction for the amplification of the 16S ribosomal RNA (16S rRNA) V3 and V4 regions. We performed 16S rRNA sequencing using the MiSeq system. The relative abundance of different bacterial taxa at the genus level in each sample has been shown. **(B)** Comparative analysis of the taxonomic composition of the fecal microbial community at the genus level. Relative abundance of the genera has been shown as a percentage.

treatment-independent manner as previously reported (3). Second, anti-inflammatory responses leading to the regression of cap polyposis might be induced by antibiotic treatment.

Mucosal prolapse syndrome and cap polyposis share endoscopic findings in that reddish elevated mucus-covered lesions are common to both conditions (4). Histopathologically, both disorders are characterized by findings of superficial erosions covered by inflammatory granulation tissue, distorted and elongated glands, and fibromuscular obliteration of the lamina propria—all these being typical findings observed in our patient. These clinical and histopathological similarities lead us to the conclusion that MPS and cap polyposis share common pathogenetic mechanisms—mucosal prolapse secondary to impaired colonic motility noted in MPS has been considered a possible etiological factor in cap polyposis. However, MPS and cap polyposis differ in terms of treatment because suppression of a chronic inflammatory response using steroid or infliximab is often effective only in the latter (2, 3). Moreover, antibiotic-induced eradication of *H. pylori* or as yet unidentified bacteria can lead to regression of cap polyposis (5, 6). These reports strongly indicate the role of chronic immune reactions toward intestinal microflora in the pathogenesis of cap polyposis. Therefore, cap polyposis might be caused by impaired host-bacterial mutualism. We have demonstrated that antibiotic treatment leads to regression of cap polyposis through significant alterations in fecal microbiota composition. Our results strongly support the idea that impaired host-bacterial mutualism caused by dysbiosis underlies the pathogenesis of cap polyposis. Further studies are warranted to assess the intestinal microbiota composition in a larger number of samples with cap polyposis to validate our results.

Significant changes in fecal microbiota composition were observed at the genus levels pre- and post-antibiotic treatment. The relative abundance of *Blautia*, *Dorea*, and *Sutterella* was markedly decreased 6 months post-antibiotic treatment compared to the pre-antibiotic treatment finding, whereas the relative abundance of *Fusobacterium* was markedly increased post-antibiotic treatment. Thus, disappearance of *Blautia*, *Dorea*, and *Sutterella* and colonization of *Fusobacterium* were observed to be associated with regression of cap polyposis. Therefore, *Blautia*, *Dorea*, and *Sutterella*, and *Fusobacterium* might be considered pathogenic and beneficial bacteria, respectively, for cap polyposis. Consistent with these results, Nishino et al. have shown that mucosal microbiota composition in those with ulcerative colitis is characterized by a greater abundance of *Blautia* (10). An increased abundance of *Dorea* has been detected in the normal colonic mucosa in patients with colorectal adenomas (11). However, an increased percentage of *Blautia* and *Dorea* observed in the gut mucosal microbiota composition is reportedly associated with remission after surgery in IBD patients (12, 13). Mukhopadhya et al. have reported that *Sutterella* is unlikely to play a role in the pathogenesis of IBD (14). Moreover, *Fusobacterium*, which demonstrated higher relative abundance post-antibiotic treatment in this patient, has been shown to promote colorectal tumor growth (15). Although the discrepancy between our data and previous reports remains unexplained, it could be attributed to a likely difference between fecal and mucosal microbiota composition. Identification of pathogenic or beneficial bacteria for cap polyposis requires future studies that assess microbiota composition in a larger number of patients with cap polyposis. Fecal microbiota analyses in a large number of patients with cap polyposis are necessary to confirm the involvement of dysbiosis and to determine the sensitivity to antibiotic treatment in this disorder.

CONCLUDING REMARKS

To our knowledge, this is the first report describing intestinal microbiota analysis in a patient with cap polyposis. Our results strongly suggest that cap polyposis may originate from dysbiosis and that microbiome-targeted therapy may be useful in this disorder.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of a Review Board of the Kindai University Faculty of Medicine with written informed consent from the patient. The patient gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by a Review Board of the Kindai University Faculty of Medicine.

AUTHOR CONTRIBUTIONS

KO, TW, YK, AO, HS, and KF took care of the patient. KO, TW, KM, KK, KY, MT, SH, and TS wrote the manuscript. TT performed pathological examinations. KO, TW, and NN performed experiments. MK supervised the research. All the coauthors checked the final version of the manuscript before the submission.

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Bacteroides fragilis Protects Against Antibiotic-Associated Diarrhea in Rats by Modulating Intestinal Defenses

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Antibiotic-associated diarrhea (AAD) is iatrogenic diarrhea characterized by disruption of the gut microbiota. Probiotics are routinely used to treat AAD in clinical practice; however, the effectiveness and mechanisms by which probiotics alleviate symptoms remain poorly understood. We previously isolated a non-toxic *Bacteroides fragilis* strain ZY-312, which has been verified to be beneficial in certain infection disorders. However, the precise role of this commensal bacterium in AAD is unknown. In this study, we successfully established an AAD rat model by exposing rats to appropriate antibiotics. These rats developed diarrhea symptoms and showed alterations in their intestinal microbiota, including overgrowth of some pathogenic bacteria. In addition, gastrointestinal barrier defects, indicated by compromised aquaporin expression, aberrant tight junction proteins, and decreased abundance of mucus-filled goblet cells, were also detected in ADD rats compared with control animals. Of note, oral treatment with *B. fragilis* strain ZY-312 ameliorated AAD-related diarrhea symptoms by increasing the abundance of specific commensal microbiota. Interestingly, we demonstrated that these changes were coincident with the restoration of intestinal barrier function and enterocyte regeneration in AAD rats. In summary, we identified a potential probiotic therapeutic strategy for AAD and identified the vital roles of *B. fragilis* strain ZY-312 in modulating the colonic bacterial community and participating in microbiota-mediated epithelial cell proliferation and differentiation.

Keywords: *Bacteroides fragilis*, antibiotic-associated diarrhea, gut dysbiosis, intestinal barrier function, enterocyte regeneration

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INTRODUCTION

Antibiotic-associated diarrhea (AAD) refers to iatrogenic diarrhea associated with antibiotic therapy and most frequently affects in-patients, especially the elderly (≥65 years), treated with broad-spectrum antibiotics (1). The clinical manifestations of AAD usually include mild and self-limiting diarrhea. It has been reported that 15–39% of AAD cases are caused by excessive growth of opportunistic pathogens, including *Clostridium difficile*, which can result in pseudomembranous colitis or a toxic megacolon, contributing to high fatality rates (2). In addition, diarrhea triggered by antibiotic use is associated with impaired resistance to pathogens as a result of the disruption of the

gut microbial flora and subsequent changes in the metabolism of carbohydrates, short-chain fatty acids, and bile acids (3).

Probiotics are defined as live microorganisms that confer health benefits to the host when administered in adequate amounts (4). As AAD results from disruption of the commensal gut microbiota due to antibiotic therapy, administration of probiotics is a reasonable therapeutic strategy to regulate or restore the gut microbiota (5). A previously published meta-analysis showed that most probiotics significantly reduce the risk of AAD in the general (mainly adult) population (6). These impressive effects have motivated many healthcare institutions to consider routine probiotic co-administration along with antibiotic treatments. However, there is still controversy regarding the usefulness of routine probiotic administration. Results of the PLACIDE trial, the largest randomized controlled trial ($n = 2941$) to date and conducted across five centers in England and Wales, showed that 21 days of treatment with a combined preparation of *Lactobacillus* and *Bifidobacterium* did not reduce the risk of either AAD or *C. difficile*-associated diarrhea (7). However, because the study was limited to only two bacterial genera among the multitude of non-pathogenic, potentially beneficial bacteria, definitive conclusions cannot be drawn regarding the benefits of probiotics for the treatment or prevention of AAD—that is, these two bacteria may not be sufficient to tip the balance of a diverse gut ecosystem, and other taxa or probiotic mixtures may be beneficial (8).

Gram-negative *Bacteroides* species are among the earliest colonizing and most abundant constituents of the gut microbiota (9). Among the *Bacteroides* species, *Bacteroides fragilis* is an important obligate anaerobe that colonizes the mammalian lower gastrointestinal tract (10). Two subtypes of *B. fragilis* have been identified and are referred to as non-enterotoxigenic *B. fragilis* (NTBF) and enterotoxigenic *B. fragilis* (ETBF). Recent studies identified the pathogenicity of ETBF with the ability to cause diarrheal disease in animals, children, and adults (11). Conversely, NTBF strains have been proposed as possible probiotics, with the potential to quell colonic inflammation. Mazmanian et al. (12–14) conducted a series of studies to demonstrate that polysaccharide A produced by *B. fragilis* strain NCTC 9343 induces an anti-inflammatory milieu involving the stimulation of interleukin-10-producing $CD4^+Foxp3^+$ T-regulatory cells in the intestine, thereby reducing pathological gastrointestinal symptoms in a mouse model of colitis. They also suggested the therapeutic potential of *B. fragilis* strain NCTC 9343 for autism spectrum disorder, whereby alteration of the gut permeability and microbial composition may ameliorate related behavioral abnormalities (15). Kasper et al. (16) also showed that *B. fragilis* strain NCTC 9343 can protect against neuroinflammation in mouse models of multiple sclerosis. Collectively, these studies raised the possibility that non-enterotoxigenic *B. fragilis* may be crucial for the establishment of beneficial intestinal microbiota and could be developed into a probiotic therapy.

We previously isolated NTBF *B. fragilis* strain ZY-312 (ZY-312) from the feces of a healthy, breast-fed infant and confirmed that it does not contain any potential virulence factors or transferable antibiotic resistance genes (17, 18). We hypothesized that this strain may alleviate AAD-related gastrointestinal symptoms and correct any associated gut ecosystem abnormalities, thereby representing a promising candidate for the prevention and/or treatment of AAD. To test this hypothesis, a stable and reproducible model of AAD must first be established. Most previous AAD models have been induced by certain pathogenic bacteria (19). However, given that *C. difficile*-associated diarrhea only accounts for 10–25% of AAD cases, we established a novel AAD rat model induced by repeated oral administration of ampicillin, streptomycin, and clindamycin. This model effectively induced the development of soft or watery stools in rats, as well as defects in intestinal integrity and alterations in the composition of commensal microbiota. We used this model to examine the therapeutic effects of ZY-312 on AAD and to explore the underlying mechanisms. Specifically, the bacterial composition and diversity in the gut were examined by 16S rRNA gene sequencing and bacterial culture in model rats gavaged with various doses of ZY-312, commercial probiotic Bifico, or a combination of Bifico and ZY-312. In addition, gastrointestinal barrier function was assessed at multilevels by quantitative real-time polymerase chain reaction assays (qRT-PCR), western blotting, immunofluorescence, and immunohistochemistry. The results of this study confirm the potential application of *B. fragilis* ZY-312 as a novel probiotic for patients with AAD.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were approved by Nanfang Hospital Animal Ethics Committee (protocol # NFYY-2014-123), in accordance with relevant ethical principles and guidelines set by the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals. Experiment involving isolation of *B. fragilis* strain ZY-312 from infant fecal was approved by the Medical Ethics Committee of NanFang Hospital (NFEC-2014-040).

Animals and the AAD Model

Sprague-Dawley rats weighing 230–250 g were purchased from the Experimental Animal Center of Southern Medical University (Guangzhou, China) and housed under specific pathogen-free conditions. The AAD model was induced by gavage daily with 2 ml of normal saline containing a mixture of clindamycin (Hisoar Pharmaceutical, Zhejiang, China), ampicillin (Jianmin Pharmaceutical Group Co., Ltd., Wuhan, China), and streptomycin (Lukang Pharmaceutical Co., Shandong, China) at various doses: low dose (combination of 25 mg/ml clindamycin, 27.75 mg/ml ampicillin, and 13.88 mg/ml streptomycin); middle dose (combination of 50 mg/ml clindamycin, 55.5 mg/ml ampicillin, and 27.75 mg/ml streptomycin); high dose (combination of 75 mg/ml clindamycin, 83.25 mg/ml ampicillin, and 41.63 mg/ml streptomycin). The antibiotics were gavaged for 7 days, and the doses were determined to be 1.35, 2.70, and 4.05 times the maximum human equivalent dose, respectively, based on previous studies (20, 21). Weight, water intake, and the presence of diarrhea were measured every second day during the experimental period. Fecal samples were collected every 3 days for microflora analyses. On days 8 and 14, half of the rats in each group were

euthanized, and 2-cm-long samples of colon tissue 3-cm distal to the anus were collected for general histopathological analyses.

Diarrhea Assessment

Diarrhea symptoms were assessed using three parameters: fecal consistency, fecal output weight in 120 min, and fecal water content, as described previously (19, 22). Fecal consistency was classified based on the following visual grading scale: (1) formed, stool maintains its shape, brown, score = 1 (**Figure 1B**, upper left panel); (2) semi-formed or soft, does not pour, yellow, score = 2 (**Figure 1B**, upper middle panel); and (3) liquid, pours more easily, yellow, score = 3 (**Figure 1B**, upper right panel). Fecal output was determined by measuring cumulative stool weight (mg) over a 120-min period. Fecal samples were weighed and dried, and then the dried solid content and total fecal content were measured. The fecal water content was calculated as follows: fecal water content = 1 − (dried solid content)/(total fecal content).

Bacteria Culture

Bacteroides fragilis strain ZY-312 was isolated from the feces of a healthy, breast-fed infant as reported (17). And the safety evaluation of ZY-312 was done previously (18). Bacteria were cultured in sterile tubes containing 10 ml of tryptone soy broth supplemented with 5% fetal bovine serum and incubated anaerobically at 37°C for 24 h in an anaerobic cabinet (Mart, Drachten, The Netherlands).

Live Bacterial Culture

Fresh feces were homogenized (10% w/v) and serially diluted in *Brucella* broth (Hope Biotechnology, Qingdao, China). Aliquots were then spread onto different selective agar plates (10^{-2} to 10^{-9} dilutions). Mannitol salt agar, BBE agar, TPY medium (Hope Biotechnology), eosin-methylene blue medium (Oxoid, Hampshire, UK), and CDC Anaerobic Agar were used to detect *Staphylococcus aureus*, *Bacteroides*, *Bifidobacterium*, Enterobacteriaceae, and *Peptococcus*, respectively. *Lactobacillus* selective agar supplemented with 1.32 ml/l glacial acetic acid was used to select *Lactobacillus* species, while CATC agar and DRBC agar were used to isolate fecal *Enterococcus* and Saccharomycetes. Reinforced *Clostridium* agar supplemented with egg was used for *Clostridium* detection. The plates were incubated at 37°C in an anaerobic cabinet or aerobic atmosphere as appropriate for 48 h.

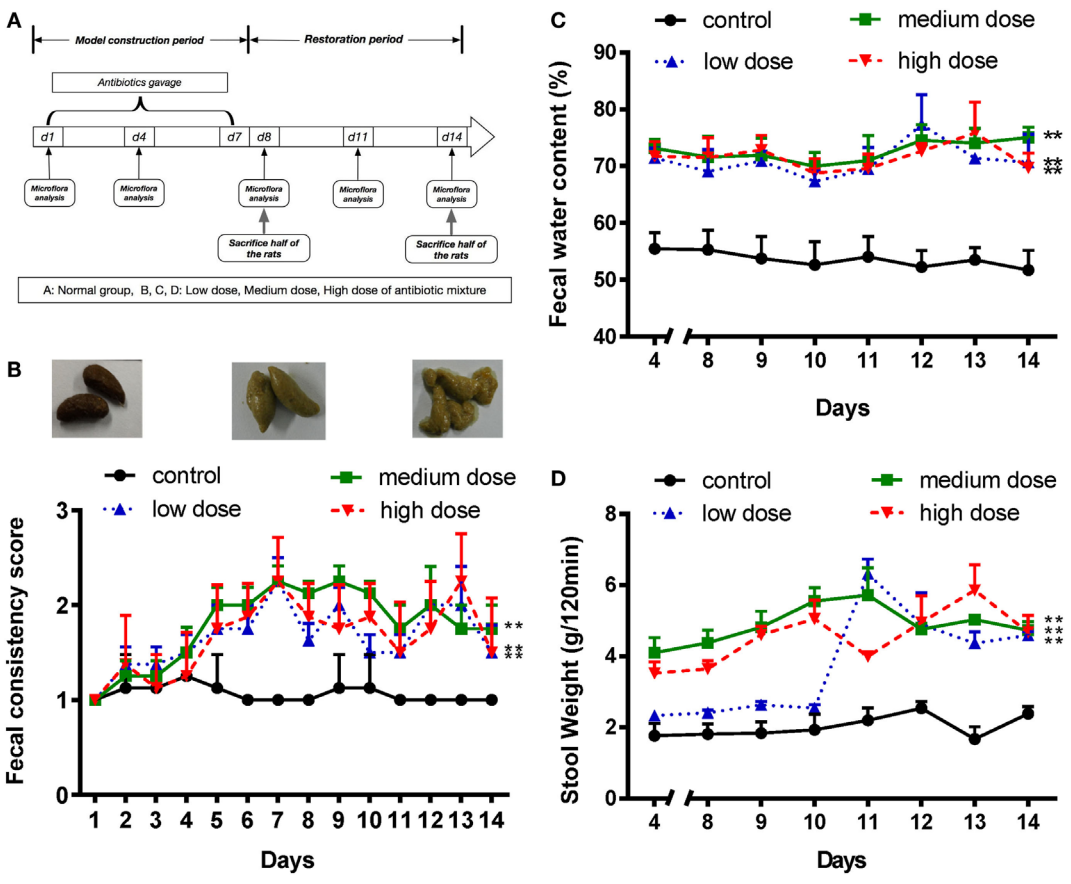

FIGURE 1 | An appropriate mixture of antibiotics induces diarrhea in SD rats. **(A)** A schematic of antibiotic-associated diarrhea model. Rats were gavaged daily with antibiotics mixture for 7 days. **(B)** Fecal consistency was assessed and classified by three visual grading scale (upper panels), and fecal consistency scores were shown in all different groups (lower panel). **(C,D)** Fecal water content and 120-min stool weight were measured in rats treated with normal saline or different doses of antibiotic cocktails (low, medium, and high), from day 4 to day 14, $n = 8$/group. Repeated measures ANOVA, $^{*}P < 0.05$ and $^{**}P < 0.01$ versus normal group.

Treatment of AAD Rats With *B. fragilis* ZY-312

During the treatment period, AAD rats were gavaged daily with 2 ml of sterile normal saline containing three different doses of *B. fragilis* ZY-312 [10^7, 10^8, or 10^9 colony-forming units (CFU)], Bifico (70 mg, SINE, Shanghai, China, containing 10^8 CFU of freeze-dried *Bifidobacterium longum*, *Lactobacillus acidophilus*, and *Enterococcus faecalis*), or a combination of Bifico and *B. fragilis* ZY-312 (10^8 CFU). A placebo group was gavaged with saline, while healthy, untreated animals served as the normal control group. For quality control, collection and administration of bacteria was finished within half an hour.

Following pre-challenge with the antibiotic mixture for 7 days (day 1–7), rats were treated as described above for 4 days (day 8–11) or 7 days (day 8–14). Diarrhea symptoms, body weight, and water intake were recorded daily. On day 11 (in the middle of treatment) and day 17, half of the animals were euthanized, respectively. Colons were collected as described above, and dissected longitudinally using sterile tissue scissors. Fecal matter (~200 mg) from the colon of each rat was then collected in 1.5-ml sterile tubes and frozen at −80°C until DNA extraction.

Microbial DNA Extraction and 16S rRNA Gene Sequencing

Rat fecal microbial DNA was extracted from stool samples using a QIAamp DNA Mini Kit (Cat.: 51504, QIAGEN, Hilden, Germany) following the manufacturer's instructions. The V3–V4 region of the 16S rRNA gene was then amplified from fecal DNA samples using the 341F/805R primer pair (341F: 5′-CCTACGGGNGGCWGCAG-3′, 805R: 5′-GACTACHVGGGTATCTAATCC-3′). All libraries were sequenced using the Illumina MiSeq platform (Illumina, San Diego, CA, USA) by Huayin Co., Guangdong, China.

Sequence analysis was performed in the Quantitative Insights into Microbial Ecology (QIIME, release v. 1.9.1) framework as follows (23). The 16S rRNA reads were initially screened for low quality bases and short read lengths. Paired reads were filtered for quality (Q30), and then assembled using Fast Length Adjustment of Short reads (v1.2.11). After filtering, from a total of 84 fecal samples, we obtained a total of 3,777,000 high-quality sequences. The average number of reads per sample was 44,965 (±2,992 SD). The resulting consensus sequences were de-multiplexed (i.e., assigned to their original sample) and trimmed of artificial barcodes and primers. Chimeric sequences were identified and removed using the usearch61 algorithm against the GreenGenes database (v13_8). We used the QIIME command pick_closed_reference_otus.py to cluster the clean data into operational taxonomic units (OTUs), and obtained a biom-format OTU table. In this process, sequences were clustered into OTUs by Usearch61_ref (v5.2.236) based on a 97% similarity threshold with the GreenGenes database (v13_8). The Chao diversity index and the number of observed species per sample were used as α-diversity metrics. β-diversity was calculated using unweighted UniFrac distances and represented in principal coordinate analyses (PCoA). Diversity was calculated by QIIME, and analyses were performed and visualized in R (v3.2.2) using pheatmap and ade4 packages. Linear discriminant analysis effect size was used to detect unique biomarkers (LDA score >3.5) in relative abundance of bacterial taxonomy (24).

Sequence files for all samples used in this study have been deposited in the European Nucleotide Archive under the accession number PRJEB22950, at http://www.ebi.ac.uk/ena/data/view/PRJEB22950. Analysis scripts and relevant files are available in GitHub (https://github.com/SMUJYYXB/B.fragilis_AAD).

Histological Analysis

Colon tissues were fixed in 4% (w/v) PFA and embedded in paraffin. Thereafter, 4-mm sections were cut and stained with hematoxylin–eosin. Colitis was assessed in tissue sections and was scored by a pathologist using a blinded experimental setup according to a standard scoring system: 0, no thickening of the colonic tissues and no inflammation (infiltration of lymphocytes); 1, mild thickening of tissues, but no inflammation; 2, mild thickening of tissues and mild inflammation; and 3, severe thickening and severe inflammation (13).

For goblet cell counting, the deparaffinized tissue sections were incubated in a 3% (v/v) glacial acetic acid solution for 3 min, in an alcian blue solution for 15 min, and then stained with Nuclear Fast Red (Sigma) for 5 min. Goblet cells were quantified by counting the numbers of alcian blue-positive cells in cross-sectional views of 30 colonic crypts per rat.

For immunofluorescence staining, deparaffinized tissue sections were steamed in citrate buffer for antigen retrieval, and blocked using phosphate-buffered saline (PBS) containing 1% (w/v) bovine serum albumin (BSA). The rabbit-derived primary antibody anti-ZO-1 (Cat.: 402200; ThermoFisher Scientific, Carlsbad, CA, USA) was used at a dilution of 1:100, and the secondary goat anti-rabbit antibody conjugated to AlexaFluor 488 was used at a dilution of 1:2,000.

For immunohistochemistry staining, the paraffinized sections were deparaffinized, rehydrated, and heat-induced antigen retrieval was performed in citrate buffer. PBS containing 1% (w/v) BSA was used to block the tissues. Primary antibodies anti-MUC2 (Cat.: 134119; Abcam), anti-Ki67 (Cat.: 12202s; CST), and anti-p-extracellular signal-related kinase (ERK) (Cat.: 4370; CST) were used at dilutions of 1:100, 1:500, and 1:200, respectively. Biotinylated secondary antibodies were purchased from ZSGB-BIO (China). Staining was visualized using 3,3′-diaminobenzidine substrate.

All samples were imaged using an Olympus fluorescence microscope (BX53; Tokyo, Japan).

Tissue RNA Extraction and qRT-PCR Assays

Total RNA was prepared from the colon tissue using TRIzol reagent (Takara, Shiga, Japan) as per the manufacturer's instructions. cDNA was generated from 1 μg of total RNA using a PrimeScript RT Reagent Kit and gDNA Eraser (Takara). Primers were designed and synthesized as previously described (5) by Sangon Biotech (Shanghai, China), and primer sequences are provided in Table S2 in Supplementary Material. qRT-PCR assays were carried out using SYBR green I Master (Roche, Basal, Switzerland) under the

following conditions: 95°C for 15 s, 94°C for 10 s, 60°C for 30 s, and 72°C for 30 s, repeated for 45 cycles.

Western Blotting

Rat colon tissues were lysed using radioimmunoprecipitation assay lysis buffer (Beyotime, Haimen, China), and tissue lysates were centrifuged at 14,000 × *g* for 30 min. Supernatants were collected and mixed with 5× sodium dodecyl sulfate (SDS) sample buffer. The samples were separated by SDS-polyacrylamide gel electrophoresis using 8–12% acrylamide gels, and then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Following incubation with primary and secondary antibodies, protein bands were detected with Immobilon Western Substrate (Millipore) and analyzed using the Bioimage analysis system (Syngene, Frederick, MD, USA). The following antibodies were used: rabbit anti-ZO-1, rabbit anti-occludin (Cat.: ab31721; Abcam), rabbit anti-phospho-ERK, rabbit anti-ERK (Cat.: 4695; CST), rabbit anti-phospho-P38 (Cat.: 4511S; CST), rabbit anti-P38 (Cat.: 8690; CST), rabbit anti-phospho-c-Jun N-terminal kinase (JNK) (Cat.: 4668; CST), and rabbit anti-JNK (Cat.: 9252; CST).

Statistical Analysis

Data are presented as means ± SEMs, unless otherwise indicated. All experiments were performed in triplicate or greater, and data are representative of five or more independent experiments. Statistical analysis of significant differences was performed using a two-tailed *t*-test, repeated measurement analysis of variance, or one-way analysis of variance, where appropriate. *P*-values of <0.05 were considered statistically significant.

RESULTS

Disruption of the Intestinal Microbiota by a Mixture of Antibiotics Triggers Diarrhea in Rats

Various doses of antibiotic mixture containing clindamycin, ampicillin, and streptomycin were used to induce AAD in a rat model (**Figure 1A**). Compared with the control group, the AAD groups showed no significant differences in body weight or food intake (Figures S1A,B in Supplementary Material). However, water intake increased significantly ($P<0.01$) in the AAD groups in a dose-dependent manner (Figure S1C in Supplementary Material). Notably, rats with AAD exhibited obvious diarrhea, as measured by fecal consistency, fecal water content, and 120-min stool weight (22, 25). Loose or liquid stools were first observed in the AAD rats on day 4, with symptoms most obvious on days 7 and 8, and lasting up to day 14 (**Figure 1B**). In addition, the fecal water content substantially increased in all AAD groups from day 4 to day14 (**Figure 1C**). The 120-min stool weight increased steadily in the medium dosage group and was significantly higher than that of the control group after day 4. In the high dosage group, parameter values fluctuated dramatically, while no significant difference was observed for the low dosage group until day 11 (**Figure 1D**). These results indicated that long-term gavage of antibiotics caused diarrhea in rats, although the severity of symptoms was not dose-dependent. Furthermore, histological examination of the intestinal tissues revealed no obvious inflammation in rats from any of the antibiotics-challenged groups (Figures S1D–F in Supplementary Material).

To examine whether these symptoms were associated with the disruption of intestinal flora, bacterial culture-based assays were performed. Results showed that the intestinal flora of the control group remained relatively stable throughout the experimental period (**Figure 2A**; Figure S2A in Supplementary Material). By contrast, in the AAD groups, decreases in the abundance of some bacterial genera, including *Lactobacillus* and *Clostridium*, were observed on day 4, and these populations had not recovered by day 14. In addition, *Bacteroides*, *Enterococcus*, *S. aureus*, and *Peptococcus* were inhibited on day 4, but these populations recovered to their pre-challenge levels (or even higher) by day 11. Interestingly, increases in the abundance of Enterobacteriaceae and Saccharomycetes were observed in the AAD groups on days 4 and 8, respectively, and these populations were still present at increased levels on day 14 (**Figures 2B**; Figure S2B in Supplementary Material). However, there were no statistically significant differences among groups treated with different doses of the antibiotics. Together, these findings indicated that the mixture of antibiotics did cause disruption of the intestinal microbiota. These changes fluctuated over time, and had not been restored to their pre-challenge levels by day 14.

These findings suggest that disruption of the microbiota by a mixture of antibiotics makes conventional rats susceptible to AAD. As a dose-dependent effect of the antibiotics was not evident, we used the medium dose of antibiotic cocktail to induce AAD in subsequent assays.

B. fragilis ZY-312 Treatment Reduces AAD-Related Gastrointestinal Symptoms

To test the effects of *B. fragilis* ZY-312 treatment in the rat AAD model, we treated AAD rats with various doses of ZY-312, Bifico, or a combination of Bifico and ZY-312. A schematic diagram of the experimental design is shown in **Figure 3A**. There were no significant differences in weight or food intake among groups (data not shown). However, while water intake remained elevated in most groups, the water intake of AAD rats treated with 10^9 CFU of ZY-312 returned to normal levels on days 12 and 13 (Figure S3A in Supplementary Material). Most rats in the AAD group treated with 10^9 CFU of ZY-312 produced formed, solid stools after day 12, and the fecal consistency score in this group decreased significantly ($P < 0.01$) compared with the AAD group. Similar results were not observed in the other treatment groups or the AAD group until day 14 (**Figure 3B**). In line with the fecal consistency results, fecal water content in the AAD group treated with 10^9 CFU of ZY-312 significantly decreased ($P < 0.01$) compared with the AAD group after day 12, while the fecal water content of other treatment groups was not significantly reduced (**Figure 3C**). Similarly, the 120-min stool weight in the 10^9 CFU ZY-312 treatment group decreased by day 12, while those of other groups were not reduced until day 14 (**Figure 3D**).

Therefore, treatment with a sufficient amount of *B. fragilis* ZY-312 appeared to have a protective effect in AAD rats, which

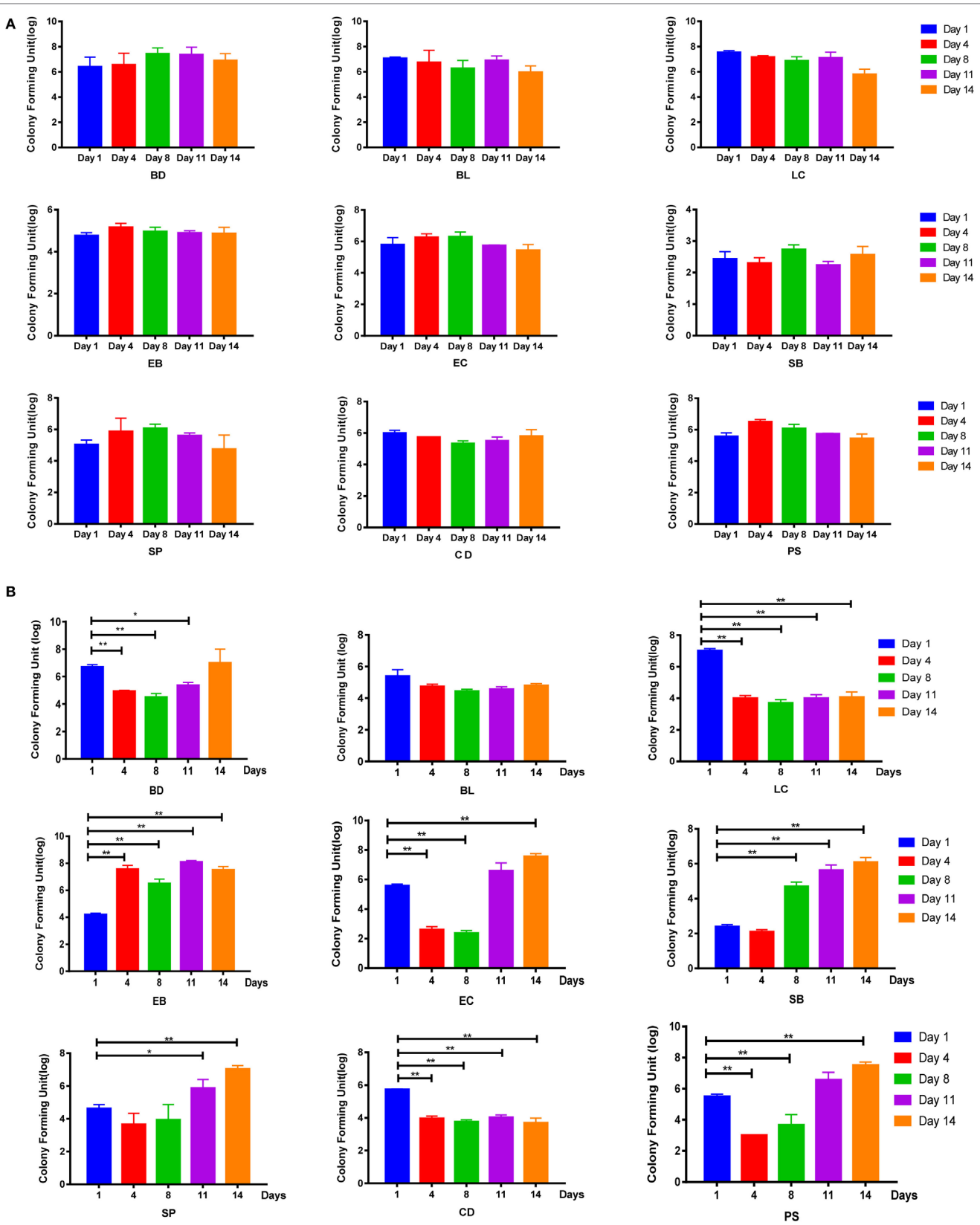

FIGURE 2 | Continued

FIGURE 2 | Disruption of SD rats microbiota by a mixture of antibiotics. **(A,B)** Bacterial culture for microbial community composition analysis of normal control rats and antibiotic-associated diarrhea rats induced by the medium dose of antibiotic cocktail. Abbreviations: BD, *Bacteroides* (in BBE agar); BL, *Bifidobacterium* (in TPY medium); LC, *Lactobacillus* (in *Lactobacillus* selective agar); EB, *Enterobacteriaceae* (in eosin-methylene blue medium); EC, *Enterococcus* (in CATC agar); SB, Saccharomycete (in DRBC agar); SP, *Staphylococcus aureus* (in Mannitol salt agar); CD, *Clostridium* (in reinforced *Clostridium* agar); PS, *Peptococcus* (in CDC anaerobic agar). *t*-Test, $^{*}P < 0.05$ and $^{**}P < 0.01$ versus day 1.

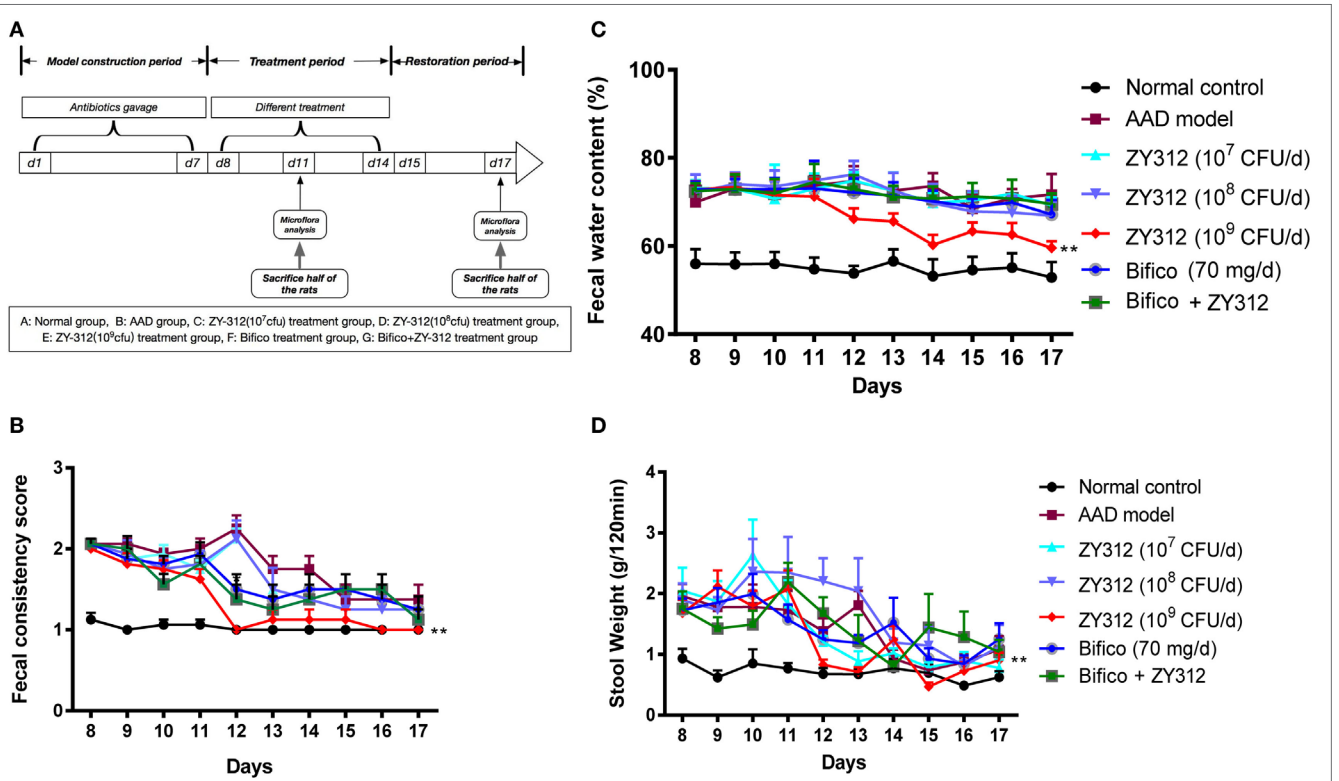

FIGURE 3 | *Bacteroides fragilis* ZY-312 improves antibiotic-associated diarrhea (AAD)-related gastrointestinal symptom. **(A)** Experimental design schematic of different treatments in AAD rats. Rats were pretreated with antibiotic mixtures for 7 days, followed by bacterial treatment. During the treatment period (day 8–14), rats were gavaged with normal saline, ZY-312 [10^7, 10^8, and 10^9 colony-forming units (CFU)/day], Bifico (70 mg/day), or Bifico (70 mg/day) combined with ZY-312 (10^8 CFU). **(B–D)** Fecal consistency scores, fecal water content, and 120-min stool weight of rats in each group from day 8 to day 17 were shown. $n = 16$/group. Repeated measures ANOVA, $^{*}P < 0.05$ and $^{**}P < 0.01$ versus AAD group.

encouraged us to further investigate the mechanism(s) underlying these effects.

B. fragilis ZY-312 Treatment Modulates Specific Microbial Changes in AAD Rats

As dysbiosis of the intestinal microbiota is a characteristic of AAD, we next investigated whether *B. fragilis* ZY-312 modulates the composition of the microbial community, thereby providing a protective effect against AAD. 16S rRNA gene sequencing was used to evaluate specific microbial alterations in all groups on days 11 and 17. Based on estimates of alpha-diversity, the total bacterial community in AAD rats was decreased on day 11 compared with the normal control group, but recovered slightly by day 17 (**Figure 4A**, left panel). In addition, unweighted UniFrac analysis revealed that the overall composition of the fecal microbial community of AAD rats was significantly different from that of normal controls on day 11, but that the difference between both groups appeared to be smaller on day 17 than on day 11 (**Figure 4A**, right panel). However, no significant differences were observed in the evenness or richness among groups of ZY-312-treated or Bifico-treated AAD rats (Figure S4A in Supplementary Material). Unweighted UniFrac analysis also demonstrated that none of the treatment groups clustered distinctly from the AAD group on either day 11 or day 17 based on PCoA (Figure S4B in Supplementary Material).

We also evaluated differences in the relative abundance of different taxa among groups. On day 11, the AAD group gut microbiota was mainly dominated by Proteobacteria, while the predominant phylum in control rats was Firmicutes. In addition, the bacterial community composition in the AAD rats had not reverted to the control composition by day 17. Interestingly, although we did not detect obvious differences among the AAD group and the treatment groups on day 11, there was a significant

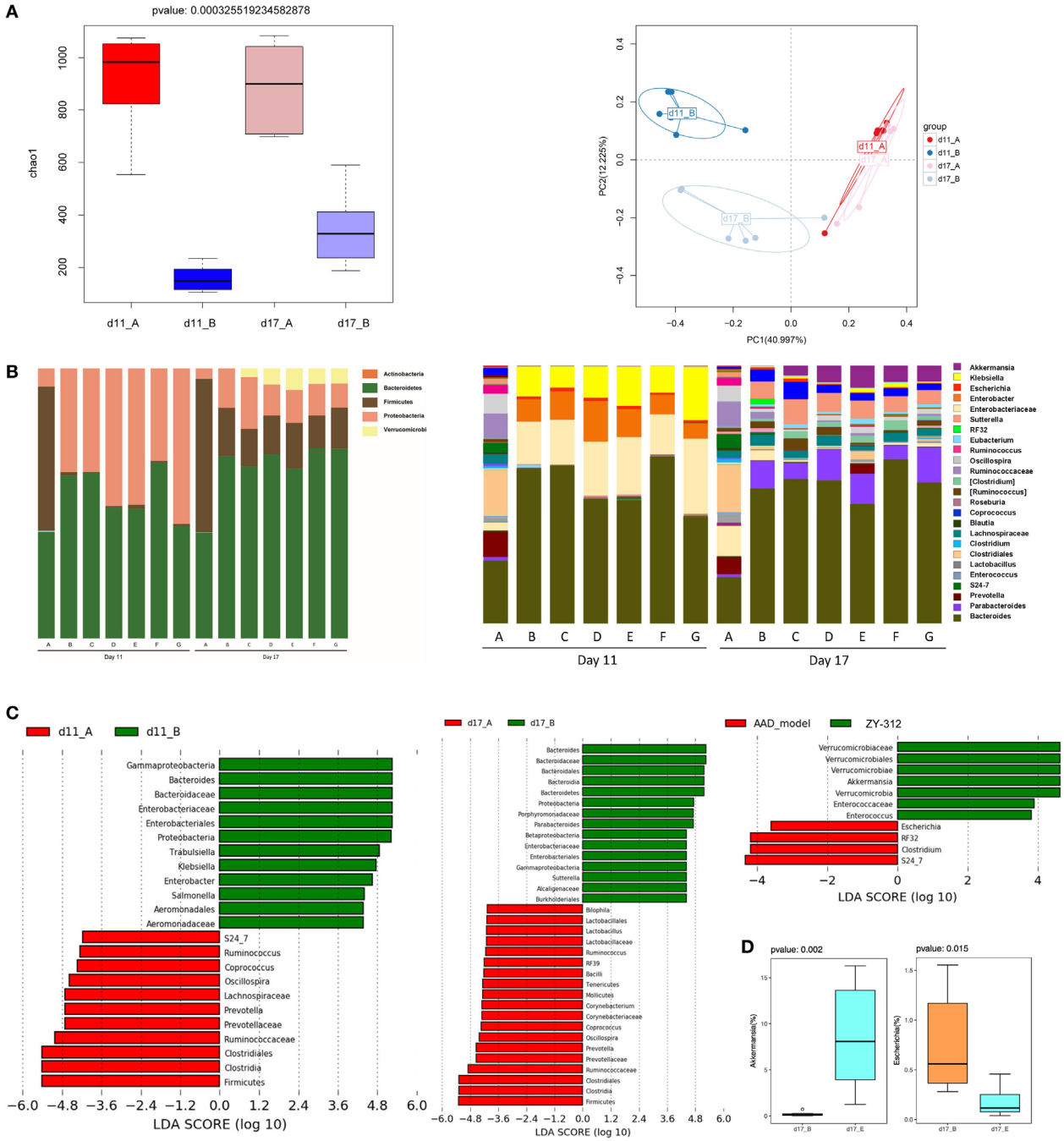

FIGURE 4 | *Bacteroides fragilis* ZY-312 restores specific microbiota changes in antibiotic-associated diarrhea (AAD) rats. **(A)** Microbial richness based on the Chao1 index, and unweighted UniFrac principal coordinate analyses based on all operational taxonomic units (OTUs) of normal rats (group A) and AAD rats (group B) on days 11 and 17, respectively. **(B)** Relative abundance of OTUs of the gut microbiota in all groups, including normal control (group A) and AAD groups gavaged with normal saline (group B), different doses of ZY-312 (10^7, 10^8, and 10^9 CFU) (group C, D, and E), Bifico (70 mg/day) (group F), or Bifico (70 mg/day) combined with ZY-312 (10^8 CFU) (group G) by gavage, at the rank of phylum (left panel) and genus (right panel). **(C)** Linear discriminant analysis effect size identifies differential abundance of bacteria between normal rats (group A) and AAD rats (group B) on days 11 and17, or between AAD rats (group B) and ZY-312 (10^9 CFU) rats (group E) (LDA > 3.5), respectively. **(D)** Relative abundance of OTUs of the genus *Akkermansia* and *Escherichia* that are significantly altered by ZY-312 treatment (group E).

increase in the relative abundance of the phylum Verrucomicrobia in the treatment groups on day 17 (**Figure 4B**, left panel).

At the genus level, four genera of beneficial microorganisms, *Oscillospira, Prevotella, Ruminococcus*, and *Coprococcus*, which include species involved in short-chain fatty acid production, were decreased or even eliminated in AAD rats on day 11, and had not been restored to control group levels on day 17. The genera *Enterobacter*, *Klebsiella*, *Trabulsiella*,

Salmonella, and *Escherichia*, all of which demonstrate pathogenic characteristics, were enriched in AAD rats on day 11. By day 17, the relative abundance of the genera *Enterobacter*, *Salmonella*, and *Klebsiella* had returned to levels similar to the control, although there was an increase in the abundance of *Sutterella* (**Figure 4B**, right panel and **Figure 4C**). In addition, although observed changes in the abundance of *Oscillospira*, *Ruminococcus*, *Coprococcus*, *Klebsiella*, and *Enterobacter* in the treatment groups were similar to those of AAD rats, and no significant differences were found among treatment groups (**Figures 4B,C**, right panels), our findings showed that one beneficial microorganism, *Akkermansia*, belonging to the phylum Verrucomicrobia, increased significantly ($P = 0.002$) in the 10^9 CFU ZY-312 treatment group on day 17, while a significant decrease ($P = 0.015$) was observed in the abundance of *Escherichia* (**Figure 4C**, right panel and **Figure 4D**).

Together, these results demonstrate that dramatic overgrowth of *Klebsiella* and *Enterobacter*, and loss of *Oscillospira*, *Prevotella*, and *Ruminococcus* species, may play a role in AAD pathogenesis. The results also confirmed that *B. fragilis* ZY-312 can modulate specific components of the commensal microbiota in AAD rats.

B. fragilis ZY-312 Improves Gut Barrier Integrity in AAD Rats

The intestinal epithelium is the interface between the gut microbiota and host tissues and is an important physical barrier preventing luminal pathogens from entering the blood stream. Histologic examination showed no obvious damage or inflammation of the intestinal epithelium in any of the experimental groups (Figure S3B in Supplementary Material). We next analyzed epithelial cell integrity by examining the expression of aquaporin and tight junction proteins in the different groups to identify possible histological and molecular changes related to diarrhea. As shown in **Figure 5A**, there were twofold and onefold decreases in the number of mucus-filled goblet cells in the colon on days 11 and 17, respectively, indicating that the secretion of mucus from goblet cells may be reduced in the colons of AAD rats. Treatment with 10^9 CFU of ZY-312 resulted, on average, in a onefold increase in goblet cell numbers in the colon on both days 11 and 17 compared with the AAD group. By contrast, other treatment groups showed no significant differences. Consistent with the above-described changes, the expression of *muc2*, which is involved in the synthesis and secretion ability of goblet cells, was significantly lower in the colons of AAD rats than in control rats on day 11, and had only slightly recovered on day 17. However, the expression of *muc2* was increased in the colons of 10^9 CFU ZY-312-treated rats on both days 11 and 17. By contrast, Bifico alone or in combination with a medium dose of ZY-312 (10^8 CFU) had no significant effect on *muc2* expression compared with the AAD group (**Figure 5B**).

We next assessed the localization and expression of ZO-1 and occludin in the colon by immunofluorescence or western blotting. Compared with the control group, the arrangement of the tight junction protein ZO-1 in AAD rats was aberrant, with discontinuous focal accumulation and reduced staining intensity in the lateral membrane on both days 11 and 17. In the ZY-312 treatment group (high dose), ZO-1 was localized to the apical regions, with intact boundaries, on days 11 and 17, as evidenced by enhanced ZO-1 immunostaining in the colon compared with that in AAD rats. However, Bifico alone failed to correct the aberrant ZO-1 expression (**Figure 5C**, left panel). Western immunoblotting results confirmed that the colonic epithelial cells of AAD rats had reduced levels of occludin, but that treatment with a high dose of ZY-312 resulted in an increase in occludin on days 11 and 17 (**Figure 5D**, left panel). In line with the protein expression results, the levels of *ZO-1* (*TJP1*) and *occludin* mRNA in AAD rats were decreased to 72 and 51%, respectively, of the levels observed in controls rats on day 11. Surprisingly, the 10^8 CFU ZY-312 treatment group showed significantly enhanced *ZO-1* and *occludin* mRNA levels compared with AAD rats on day 11, while there was only a slight increase in the transcription of these genes in the 10^9 CFU ZY-312 treatment group. Bifico did not appear to affect the transcriptional levels of either *ZO-1* or *occludin*. The expression levels of the genes encoding *ZO-1* and *occludin* in AAD rats were similar to those of the controls on day 17, and there was no significant difference between the treatment groups and the AAD group at this time point (**Figures 5C,D**, right panels).

We also investigated the expression levels of genes encoding aquaporins (*Aqp*) in the colon and demonstrated that the expression of *Aqp1*, *Aqp3*, and *Aqp8* in AAD rats was reduced to 65, 86.2, and 61.1% of the levels in control rats, respectively, on day 11. On day 17, *Aqp3* and *Aqp8* mRNA levels in the AAD rats remained lower than those of the control animals, while the expression of *Aqp1* was 1.28-fold greater than that in the control group (**Figure 5E**). Surprisingly, treatment with a low dose (10^7 CFU) of ZY-312 or with Bifico was sufficient to accelerate the recovery of *Aqp1* expression, with *Aqp1* mRNA levels in these treatment groups similar to those of the control group at day 11. In addition, the levels of *Aqp3* mRNA in the 10^9 CFU ZY-312 treatment group showed a threefold increase compared with the control group at day 11, but were restored to control levels by day 17. Likewise, *Aqp8* mRNA levels in the 10^8 CFU ZY-312 treatment group were increased by more than threefold on day 11, but remained high on day 17. Notably, all doses of ZY-312 increased *Aqp8* expression, while Bifico appeared to suppress the expression of both *Aqp3* and *Aqp8* compared with the control and ADD groups on day 17. In short, appropriate ZY-312 treatment could restore the expression of *Aqp1* and enhance the expression of *Aqp3* and *Aqp8*.

Finally, to further investigate whether the above changes were associated with epithelial regeneration, we stained colonic sections with Ki67, which is a cellular marker for proliferation. We found that high doses (10^9 CFU) of ZY-312 induced a marked accumulation of Ki67-positive colonocytes along the length of the crypt and at the luminal surface (**Figure 6A**). Activation of mitogen-activated protein (MAP) kinase ERK is also known to stimulate proliferative gene regulatory events and initiate goblet cell differentiation (26). Our results showed ERK phosphorylation was decreased in AAD rats, while high doses of ZY-312 caused an increase in p-ERK on day 11 (**Figures 6B,C**). Moreover, p38 and JNK, the other two MAP kinases, which are frequently reported to trigger epithelial cellular apoptosis and inflammatory responses (27), were slightly downregulated or remained unchanged in

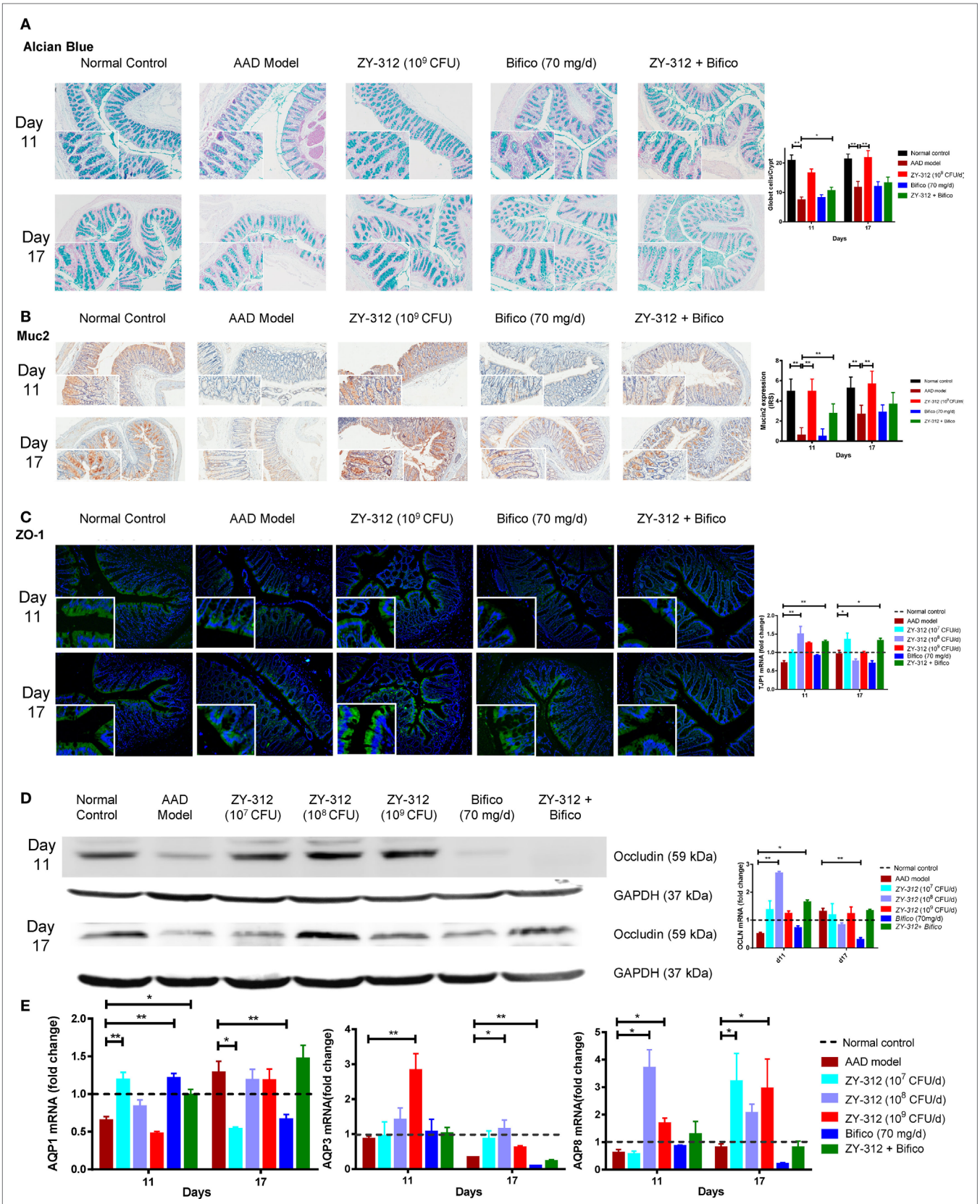

FIGURE 5 | Continued

FIGURE 5 | *Bacteroides fragilis* ZY-312 improves gut barrier integrity in antibiotic-associated diarrhea (AAD) rats. **(A)** Alcian blue staining for detecting goblet cells numbers were shown in normal rats, AAD rats treated with normal saline, ZY-312 [10^9 colony-forming units (CFU)], Bifico (70 mg/day), or Bifico (70 mg/day) combined with ZY-312 (10^8 CFU) on days 11 and 17. $n = 8$/group. **(B)** Immunohistochemistry of Muc2 protein located in colonic tissues was detected in all groups as mentioned above. **(C)** Immunofluorescence staining for ZO-1 and *ZO-1* (*TJP1*) mRNA level in all the groups was shown as mentioned above. The magnifications of the above figures are 10× and 40×. **(D)** Colon protein level and mRNA expression of occludin in all groups on days 11 and 17 were shown. **(E)** qPCR analysis of AQP1, AQP3, and AQP8 gene in colonic tissues were detected in all groups as indicated in **Figure 3**, with mRNA fold changes normalized to those of normal control group. One-way ANOVA, $^*P < 0.05$ and $^{**}P < 0.01$ versus AAD group.

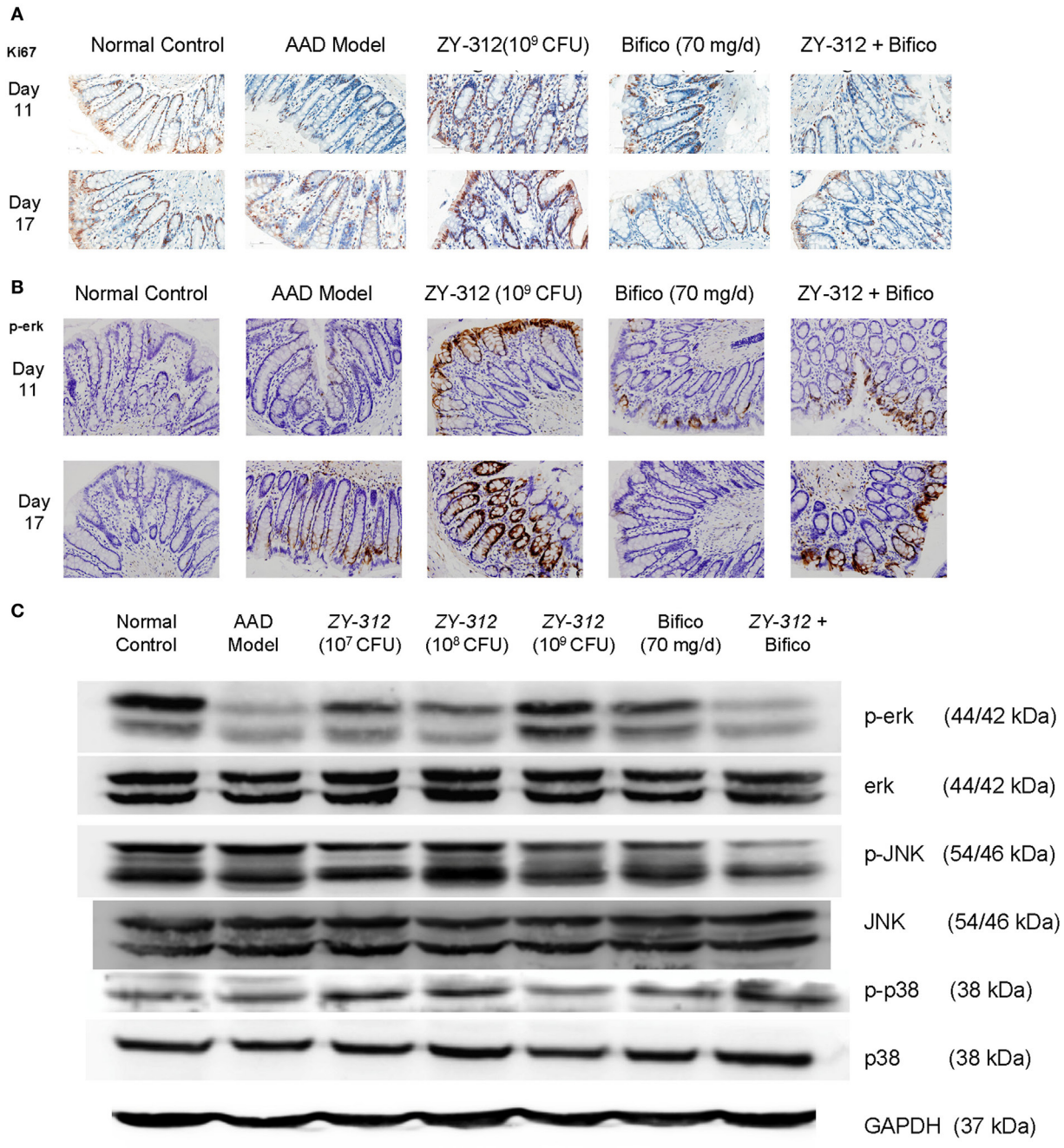

FIGURE 6 | *Bacteroides fragilis* ZY-312 activates Erk signaling and promotes proliferation in antibiotic-associated diarrhea (AAD) rats. **(A,B)** Cell proliferation marker Ki67 and p-ERK1/2 were detected by immunohistochemistry in colon sections on days 11 and 17 in all groups as mentioned in **Figure 5A**. All images were taken at the same magnification at 40×. **(C)** Phosphorylation of extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 was also detected by western blot in colon tissues on day 11 in all groups as mentioned in **Figure 5A**.

response to high doses of ZY-312 compared with AAD group (**Figure 6C**).

Taken together, these results indicate that epithelial arrangement and organization are impaired in AAD rats, demonstrated by a reduction in aquaporin expression, decreased numbers of mucus-filled goblet cells, and aberrant tight junctions, all of which contribute to the impaired mucosal barrier in AAD rats. Treatment with ZY-312 plays a role in modifying the proliferation and function of the intestinal epithelium and maintaining the integrity of the intestinal barrier, thereby alleviating symptoms of AAD in rats.

DISCUSSION

Antibiotic-associated diarrhea is defined as unexplained diarrhea associated with antibiotic therapy (28). In the present study, we successfully established an AAD model by treating conventional rats with a mixture of antibiotics, which effectively triggered diarrhea symptoms. Previous studies suggested that the main mechanisms by which antibiotics induce AAD are the disturbance of the composition and function of the normal intestinal microbiota, and/or the allergic and toxic effects of antibiotics on the intestinal mucosa (29). We used bacterial culture methods and 16S rRNA gene sequencing to confirm that AAD rats show a significant decrease in the biodiversity of the colonic microbiota, together with overgrowth of certain genera of colitis-associated bacteria, including *Enterobacter*, *Klebsiella*, *Trabulsiella*, *Salmonella*, and *Escherichia*. In particular, the genera *Escherichia*, *Klebsiella*, and *Enterobacter* are commonly associated with nosocomial infections. A recent study has shown that multidrug-resistant strains of these three genera dominate the gut microbiota of preterm infants following specific antibiotic treatments (30). Therefore, we speculate that overgrowth of certain pathogenic bacteria may be responsible for the symptoms of AAD.

A growing body of evidence suggests that consumption of probiotics may promote gastrointestinal health, presenting a new avenue of treatment for various diseases such as inflammatory bowel syndrome, obesity, and multiple sclerosis (31–33). The commercial probiotics Bifico, which consists of *B. longum*, *L. acidophilus*, and *E. faecalis*, has long been considered a routine treatment for functional diarrhea in clinical practice in China (34), but its effectiveness has not been extensively evaluated. Therefore, we also administered rats with Bifico to test its efficacy in the AAD model. To our surprise, Bifico alone did not have any effect on diarrhea symptoms, nor did it worked when combined with 10^8 CFU *B. fragilis* ZY-312, though the bacterial interaction of both remains unknown. However, an individual treatment with appropriate oral dose of *B. fragilis* ZY-312 (10^9 CFU) is sufficient to ameliorate diarrhea symptoms in AAD rats, supported by the restoration of fecal consistency, fecal water content, and 120-min stool weight. Moreover, we found that the consumption of adequate amounts of ZY-312 could alter the composition of the intestinal microbiota in AAD rats, characterized with the inhibition of overgrown *Escherichia* and promotion of the growth of the commensal bacterium *Akkermansia muciniphila*, which has been considered as protective in type 2 diabetes (35).

With intestinal microecology being a topic of intense investigation, the modulation of *B. fragilis* in gut microbiome has become a heated issue. Generally, *B. fragilis* could be divided into NTBF and ETBF. The ETBF, which produce *B. fragilis* enterotoxin (BFT), have been found to be associated with the occurrence of diarrhea diseases including AAD in human (36). The diarrheal agent BFT could not only stimulate fluid secretion in intestinal epithelial cells *via* NF-kappaB/cyclooxygenase-2 activation but also destroy cellular interactions of intestinal epithelial cells through E-cadherin cleavage (37). What is worse, ETBF infection could also contribute to chronic colitis and promote colorectal tumorigenesis in mice (36). By contrast, NTBF act the opposite way. Recent study revealed that NTBF strain NCTC 9343 are able to colonize stably deep within crypt channels following microbiome disruption with *Citrobacter rodentium* infection or antibiotic treatment, owing to their *c*ommensal *c*olonization *f*actors (*ccf*) locus. The upregulation of *ccf* genes during *B. fragilis* strain NCTC 9343 colonization would resist colonization by the same, but not different, species, which could partially account for the rule of species-specific saturable colonization in *Bacteroides* species (38). Further, additional study demonstrated that *B. fragilis* strain NCTC 9343 can restrict enteric colonization of ETBF strain *via* a type VI secretion system (T6SS) (39). Besides, our previous study showed that *B. fragilis* strain ZY-312 can shorten the colonization time of *Vibrio parahaemolyticus* in mice, and that *B. fragilis* cell lysate enhances the ability of bone marrow-derived macrophages to phagocytize pathogenic bacteria, such as enterohemorrhagic *Escherichia coli* (40, 41). To conclude, the mocrobiota modulation of NTBF in AAD may rest in suppression of enteropathogenic bacteria together with promotion of probiotics like *A. muciniphila*.

Recent data have suggested intriguing roles for the interactions between gut microbiota and epithelial cells (42). Epithelial cells, which are sealed by tight junctions and mucus from the gastrointestinal tract, form a first line intestinal barrier between the luminal contents and the host. Therefore, these cells serve as the primary gatekeepers and regulators of bacterial interactions with the host immune system (43). Aquaporins are water-channel membrane proteins expressed in various tissues, with AQP1, 3, 4, and 8 mainly expressed in the colon (44). We observed that the AAD rats exhibited defective gastrointestinal integrity and improper epithelial organization, with decreased expression of aquaporin-encoding genes, aberrant tight junction proteins, and a decrease in the number of goblet cells compared with control animals. Although these changes have not been directly linked to pathogenic bacteria, the findings suggest that a defective gut barrier provides the opportunity for pathogens to gain access to the host, which is considered the main cause of loose stools in AAD rats. Indeed, a related study showed that *B. fragilis* strain NCTC 9343 can regulate intestinal permeability and maintain metabolic homeostasis (15). Consistent with these findings, the current study demonstrated that the administration of an adequate amount of *B. fragilis* strain ZY-312 can repair the structure of intestinal barrier by increasing the proliferation of goblet cells, which are mainly responsible for mucus production, and by enhancing the expression tight junction proteins ZO-1 and occludin, which maintain the barrier between shedding

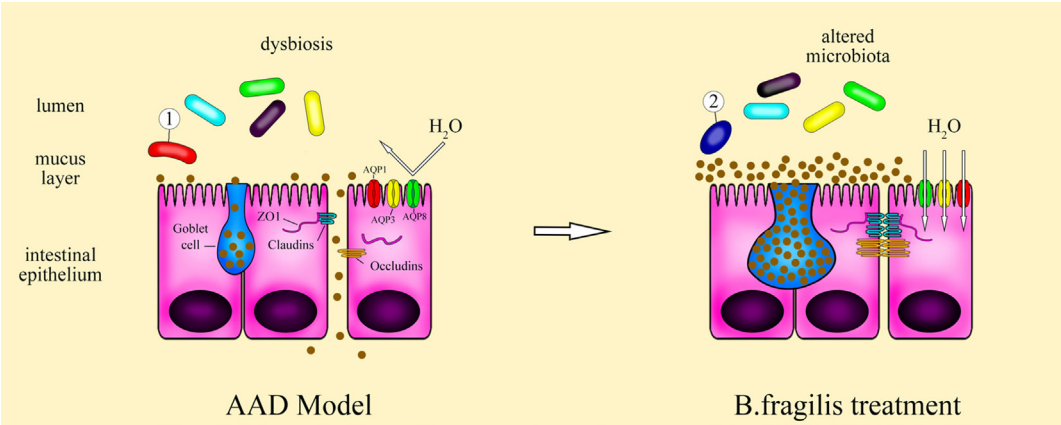

FIGURE 7 | Schematic of the therapeutic role of *Bacteroides fragilis* ZY-312 in AAD symptom. Antibiotic-associated diarrhea (AAD) rats exhibit dysbiosis of the gut microbiota, with overgrowth of some pathogenic bacteria and then resulting in defective gastrointestinal integrity and improper epithelial organization with low aquaporins, aberrant tight junction proteins, and a decreased number of mucus-filled goblet cells. Treatment of AAD rats with *B. fragilis* ZY-312 alters the composition of the gut microbiota, with promotion of commensal *A. muciniphila* growth, and subsequently remodeling of the colonic epithelium by improving enterocyte monolayer integrity, increasing mucus layer thickness, and water transport in rats.

and adjacent healthy epithelial cells. Moreover, *B. fragilis* strain ZY-312 restored *Aqp1* expression in AAD rats and increased the expression levels of *Aqp3* and *Aqp8*, all of which are responsible for water transfer and absorption in the rat colon.

Various enteric microbes regulate intestinal cell tight junctions and goblet cell differentiation. Among the bacteria affected by treatment with *B. fragilis*, two have valid functions in epithelial cell organization and barrier function: *A. muciniphila* and *E. coli*. *A. muciniphila* is a mucin-degrading bacterium that improves enterocyte monolayer integrity, increases the mucus layer thickness, and corrects the gut barrier (45, 46). Moreover, *A. muciniphila* influences epithelial cell gene expression (47) and promotes mucosal wound repair *via* FPR1-dependent redox-mediated control of epithelial cell proliferation and migration (48). In addition, early-colonizing commensal *E. coli* drive the remodeling of the colonic epithelium by affecting the structure of the epithelium, mucus layer, and ion and water transport channels in rats, whereas pathogenic strains of *E. coli* bring about the opposite effects (49, 50). However, further studies are needed to elucidate whether or not these beneficial effects are exerted directly by *B. fragilis* or indirectly *via* the regulation of other bacteria.

Several pathways are associated with epithelial cell regeneration. The ERK pathway is activated by growth factors and other mitogenic stimuli, and mediates proliferative gene regulatory events and initiates goblet cell differentiation (26, 51, 52). Some commensal bacteria initiate epithelial cell growth and wound healing *via* ERK signaling (51), including *Akkermansia* species (regulated by *B. fragilis*), which stimulates ERK phosphorylation, thereby promoting epithelial wound closure and goblet cells differentiation (26, 48). These findings are in line with our present study, which indicate that a high dose of *B. fragilis* strain ZY-312, together with an increased *Akkermansia* population, induces a marked accumulation of Ki67-positive colonocytes and increased ERK phosphorylation in AAD rats. Thus, *B. fragilis* ZY-312 may play a role in modifying the proliferation and differentiation of intestinal epithelial cells *via* ERK signaling. However, further efforts are needed to investigate the specific molecular biological processes involved.

Collectively, as shown by **Figure 7**, these results suggest that *B. fragilis* strain ZY-312 treatment can ameliorate the gastrointestinal symptoms of AAD in rats by modulating gut microbiota, thereby restoring epithelial cell organization and barrier function, which is mediated in part through ERK signaling. Further studies are required to determine the specific roles of *B. fragilis* in the intestinal epithelium and to better understand the molecular mechanism involved. Nevertheless, this study lays a clear foundation for further investigations of microbiota-modulated epithelial function.

ETHICS STATEMENT

All animal experiments were approved by Nanfang Hospital Animal Ethics Committee (protocol # NFYY-2014-123), in accordance with relevant ethical principles and guidelines set by the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals. Experiment involving isolation of *B. fragilis* strain ZY-312 from infant fecal was approved by the Medical Ethics Committee of NanFang Hospital (NFEC-2014-040).

AUTHOR CONTRIBUTIONS

WZ conducted the experiments, analyzed data, and wrote the manuscript; BZ helped with performing experiments, analyzed data, and contributed to revising the manuscript; JX helped perform experiments, revised the manuscript, and contributed to data interpretation; YL and EQ analyzed data and contributed to revising the manuscript; ZhijunL did the experiments with mice; ZhengchaoL and YH analyzed data; HZ and YB directed

the experiments and contributed to revise the manuscript; FZ provided overall directions and contributed to revise the 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/fimmu.2018.01040/full#supplementary-material.

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Commensal Bacteria-Dependent CD8αβ$^+$ T Cells in the Intestinal Epithelium Produce Antimicrobial Peptides

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The epithelium of the intestine functions as the primary "frontline" physical barrier for protection from enteric microbiota. Intraepithelial lymphocytes (IELs) distributed along the intestinal epithelium are predominantly CD8$^+$ T cells, among which CD8αβ$^+$ IELs are a large population. In this investigation, the proportion and absolute number of CD8αβ$^+$ IELs decreased significantly in antibiotic-treated and germ-free mice. Moreover, the number of CD8αβ$^+$ IELs was correlated closely with the load of commensal microbes, and induced by specific members of commensal bacteria. Microarray analysis revealed that CD8αβ$^+$ IELs expressed a series of genes encoding potent antimicrobial peptides (AMPs), whereas CD8αβ$^+$ splenocytes did not. The antimicrobial activity of CD8αβ$^+$ IELs was confirmed by an antimicrobial-activity assay. In conclusion, microbicidal CD8αβ$^+$ IELs are regulated by commensal bacteria which, in turn, secrete AMPs that have a vital role in maintaining the homeostasis of the small intestine.

Keywords: CD8αβ$^+$ intraepithelial lymphocytes, α-defensins, antimicrobial activity, intestinal epithelium, commensal bacteria

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INTRODUCTION

Intestinal epithelial cells (IECs) are a single layer of cells covering the luminal side of the intestinal tract. These cells are confronted by trillions of resident commensal bacteria (1). Intraepithelial lymphocytes (IELs) are the lymphocytes located between enterocytes. IELs have a vital role in protective immunity against invading pathogens, as well as providing tolerance to commensal bacteria, and maintaining intestinal homeostasis (2–4). These "frontline" IELs are exclusively T cells and are predominantly CD8$^+$ and can be divided into two subsets based on the expression of T cell receptors and co-receptors (3). Among the two subsets, type A IELs are conventional CD8αβ$^+$TCRαβ$^+$ or CD4$^+$TCRαβ$^+$ cells and are pre-activated in gut-associated lymphoid tissues or intestinal draining lymph nodes (LNs), before they "home" to the intestinal epithelium. These T cells show an "effector-memory-like phenotype" and have a protective role against various pathogens. Type B IELs are CD8αα$^+$ TCRαβ$^+$ or CD8αα$^+$ TCRγδ$^+$ and do not express CD4 or CD8αβ. These T cells participate in damage repair, the regulatory response, and are potent cytotoxic effectors (2, 5–12).

CD8αβ$^+$ IELs are the major type A IELs. They are the progeny of conventional CD8 T cells activated by peripheral antigens in Peyer's patches (PP) or mesenteric LNs and accumulate with age in the epithelium of mice (2, 13). Adoptive transfer of antigen-specific CD8αβ$^+$ IELs into mice infected with lymphocytic choriomeningitis virus, rotavirus, *Giardia lamblia*, or *Toxoplasma*

gondii has demonstrated a protective function for CD8αβ+ IELs in infection (14–17). CD8αβ+ IELs are strongly cytotoxic, and are a subset of antigen-experienced cytotoxic T lymphocytes (CTLs) (2). However, these IELs typically encounter a large number of commensal bacteria rather than invading pathogens, so two main questions arise: what is the effect of CD8αβ+ IELs on commensal bacteria? Do CD8αβ+ IELs function in ways other than conventional CTLs when confronted by commensal bacteria? These questions and the relationship of these IELs to commensal bacteria have not been explored fully. Comparison of wild-type, germ-free (GF), and antibiotic-treated mice has demonstrated a positive relationship between CD8αβ+ IELs and commensal bacteria, suggesting that the latter may regulate the number of CD8αβ+ IELs in the intestinal epithelium (18–20). However, the mechanistic basis for the effect of CD8αβ+ IELs on commensal bacteria is not known.

As a physical barrier between the connective tissue and intestinal lumen, the intestinal epithelium utilizes various antibacterial mechanisms to maintain a steady-state relationship between the host and bacteria. Defensins within intestinal crypts have a critical role in this relationship. Defensins are a family of antimicrobial peptides (AMPs) that can be divided into three subfamilies α-, β-, and θ-defensins, according to their structural features (21). In humans and other mammals, α-defensins are usually secreted by neutrophils, monocytes, and some epithelial cells. In mice, leukocytes do not express α-defensins but Paneth cells do, and are located in the crypts of the small intestine, where α-defensins are a predominant antimicrobial factor (21–24). Paneth cells are located at the base of crypts (which are at the bottom of villi) and release AMPs in the form of secretory granules (3). Villi extend into the lumen, and have a large surface area for contact with intestinal contents. Whether α-defensins are produced along the surface of villi is not known. However, it is possible that some lymphocytes scattered along the intestinal epithelium may also produce anti-microbial factors that help to control the large number of microorganisms within the lumen.

To study the relationship between CD8αβ+ IELs and commensal bacteria, we examined CD8αβ+ IELs in GF and antibiotic-treated mice and showed that they are dependent upon commensal bacteria. In an experiment involving microbiota transplantation, we provide evidence that CD8αβ+ IELs can be induced by some specific members of commensal bacteria. In addition, a similar decrease in the number of CD8αβ+ IELs in toll-like receptor (TLR)-deficient mice suggested commensal bacteria may regulate the number of CD8αβ+ IELs *via* TLR stimulation. We conducted microarray analysis and showed that CD8αβ+ IELs expressed a series of α-defensins at gene and protein levels. The supernatants of cultured CD8αβ+ IELs killed bacteria directly and showed antimicrobial activity. Overall, our results provide evidence for a new function by which CD8αβ+ IELs regulate commensal bacteria in the epithelium of the small intestine.

MATERIALS AND METHODS

Mice

Wild-type [specific pathogen-free (SPF) and GF] mice on a C57BL/6 background were purchased from the Shanghai Laboratory Animal Center (SLAC, Chinese Academy of Sciences). GF and corresponding SPF mice on a BALB/c background were generated and provided by Professor Hong Wei from Third Military Medical University (Chongqing, China). TLR2$^{-/-}$, TLR4$^{-/-}$, TLR9$^{-/-}$, and TLR2$^{-/-}$TLR4$^{-/-}$TLR9$^{-/-}$ mice (SPF) on a C57BL/6 background were a gift from Dr. Shaobo Su (Sun Yat-sen University, Guangzhou, China). GF mice were housed in GF facilities according to animal care regulations. SPF mice were maintained in a SPF barrier facility at the University of Science and Technology of China (USTC, Hefei, China) in accordance with the guidelines for use of experimental animals. Sex-matched mice (8–10 weeks) were used for all experiments unless indicated otherwise.

Isolation of IELs and Splenocytes (SPLs)

Intraepithelial lymphocytes were isolated as described previously (25) with modifications. The entire small intestines were extracted and placed in cold phosphate-buffered saline (PBS) after mesenteric fat tissue had been removed. After careful identification of individual PPs on the anti-mesenteric side of the intestinal serosa and excising them using surgical scissors, the small intestines were opened longitudinally and washed with PBS 4–5 times to remove most of the contents. Then, the intestines were cut into pieces (1 cm) and placed in a 50-mL Erlenmeyer flask. The intestinal pieces were incubated in 20 mL of Iscove's modified Dulbecco's medium (IMDM) supplemented with 5% fetal bovine serum (FBS), 5 mM ethylenediamine tetra-acetic acid (EDTA), and 15 mM HEPES, and rotated at 200 rpm for 30 min at 37°C. This procedure was repeated thrice. After each rotation, the supernatants containing IELs were collected, and new IMDM with EDTA and HEPES was added to the Erlenmeyer flask. The supernatants were filtered through a 200-gauge steel mesh and cells (including IELs) were collected by centrifugation. Total cells were resuspended in 3 mL of 40% Percoll and then overlaid onto 2 mL of 70% Percoll, after which gradient centrifugation was undertaken at room temperature. IELs at the interphase were collected. The cells were washed once in PBS and these IELs were used for experimentation.

Spleens were extracted and placed in cold PBS. After filtration through a 200-gauge steel mesh and the removal of red blood cells (RBC) with RBC lysis buffer, SPLs were collected for further experimentation.

Flow Cytometry and Sorting

For surface staining, IELs and SPLs were incubated with 5% normal rat serum to block FcRs and incubated with indicated antibodies for 30 min at 4°C. For intracellular staining, IELs and SPLs were pretreated with phorbol myristate acetate (PMA; 50 ng/mL; Sigma–Aldrich, Saint Louis, MO, USA), ionomycin (1 μg/mL; Sigma–Aldrich), and monensin (10 μg/mL; Sigma–Aldrich) for 5 h. Then, the stimulated IELs and SPLs were incubated with 5% normal rat serum to block FcRs and stained with surface markers. After fixation and permeabilization, cells were stained further with intracellular antibody. The antibodies used are listed in Table S1 in Supplementary Material. All flow-cytometry experiments were carried out with a flow cytometer (LSRII; BD Biosciences, Franklin Lakes, NJ, USA) and analyzed with FlowJo software

(Tree Star, Ashland, OR, USA). For microarray analysis, sorting of CD8αβ+ IELs and CD8αβ+ SPLs was undertaken with a FACS Aria cell sorter (BD Biosciences) and purity >98% was achieved.

We carried out an assay to measure antimicrobial activity. To obtain cells with increased activity, purified CD8αβ+ IELs and CD8αβ+ SPLs were sorted by magnetic-activated cell sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were labeled with biotin anti-mouse CD8b antibody and then anti-biotin microbeads. The purity of the sorted cell populations was >90%.

Antibiotic Treatment

Commensal bacteria from the gut were depleted using four antibiotics according to method reported before (26) with modifications. Briefly, for combinatorial antibiotic treatment, mice were provided with sterile water supplemented with metronidazole (1 g/L), neomycin sulfate (1 g/L), ampicillin (1 g/L), and vancomycin (0.5 g/L) (Sangon Biotech, Shanghai, China) beginning at 3 weeks of age and lasting for 5 weeks. For single antibiotic treatment, mice were treated with sterile water supplemented with each antibiotic alone at the dose indicated above. Water containing antibiotics was supplied as drinking water to mice and changed twice a week.

Analyses of Bacterial 16S rDNA

The contents of a 15-cm distal portion of small intestines were collected and weighed. Bacterial DNA was extracted using an E.Z.N.A™ Mag-Bind Soil DNA kit (Omega Bio-Tek, Norcross, GA, USA). To measure the composition of bacteria in the small intestine, the V3–V4 region of 16S rDNA was amplified and sequenced using Illumina Miseq™ (Sangon Biotech).

Transplantation With *Bifidobacterium*

The transplantation experiment was carried out according to previous reports with modifications (27, 28). Mice at 3 weeks of age were treated with a combination of metronidazole (1 g/L), neomycin sulfate (1 g/L), ampicillin (1 g/L), and vancomycin (0.5 g/L) (Sangon Biotech) for 2 weeks. Then, at 5 weeks of age, the water was replaced by antibiotic-free water and the mice were administered (i.g.) with a mixture of 2×10^8 CFU *Bifidobacterium longum* (ATCC15697) and 2×10^8 CFU *Bifidobacterium adolescentis* (ATCC15703). Also, the antibiotic-free drinking water was supplied with *B. longum* (final concentration $\approx 10^6$ CFU/mL) and *B. adolescentis* (final concentration $\approx 10^6$ CFU/mL). Mice in the control group were administered (i.g.) with an equal volume of Modified Reinforced Clostridial Medium (BD 218081) but without bacterial cells and the antibiotic-free drinking water was also supplied with an equal volume of Modified Reinforced Clostridial Medium. Mice were evaluated after 15 days.

Identification of Bacteria

Nine days after transplantation of *Bifidobacterium*, fresh stool samples were collected and weighed. To identify the existence and relative expression of *Bifidobacterium*, DNA from the stool samples was extracted using a Stool Genome DNA Extraction kit (YPH-Bio, Beijing, China) according to manufacturer instructions. The abundance of *Bifidobacterium* was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) with SYBR® Premix Ex Taq (Tli RNaseH Plus; TaKaRa Bio, Kusatsu, Japan). The relative expression of the target gene was normalized to the gene level of "all bacteria."

As reported previously (29, 30), the following primers (forward and reverse, respectively) were used: "all bacteria": 5′-CGGTGA ATACGTTCCCGG-3′ and 5′-TACGGCTACCTTGTTACGAC TT-3′; *Bifidobacterium*: 5′-CTCCTGGAAACGGGTGG-3′ and 5′-GGTGTTCTTCCCGATATCTACA-3′.

Microarray Analysis

Samples were collected from purified CD8αβ+ IELs and CD8αβ+ SPLs, with ≈1 million cells in each sample. Microarray analysis was done with PrimeView™ chips (Affymetrix, Santa Clara, CA, USA) and analyzed using MeV 4.9 software.

Real-Time Polymerase Chain Reaction

RNA purified from 1 million CD8αβ+ IELs and 1 million CD8αβ+ SPLs was isolated using TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA). Primers were synthesized by Sangon and are listed in Table S2 in Supplementary Material.

Immunofluorescence

Isolated CD8+ IELs were attached to poly-L-lysine-coated slides and then fixed for 20 min with 4% paraformaldehyde at room temperature, after which the slides attached with CD8+ IELs were stored at −80°C. Slides with IELs were moved to room temperature for 5 min and permeabilized with 1% Triton X-100 for 15–20 min, followed by blocking with 1% bovine serum albumin for 1 h at room temperature. Subsequently, slides were stained with primary antibodies at 4°C overnight. The slides were then washed thrice in PBST (PBS with 0.05% Tween 20) for 5 min. Slides were incubated with secondary antibody in the dark for 2 h at 37°C and then stained with 4′,6-diamidino-2-phenylindole (1 ng/mL) for 3 min at room temperature. After a final wash in PBST, the slides were mounted on coverslips. Cells were imaged with a microscope (LS710; Zeiss, Wetzlar, Germany). Detailed information about antibodies is listed in Table S1 in Supplementary Material.

Stimulation of Secretion of CD8αβ+ T Cells

CD8αβ+ IELs (5×10^5) and CD8αβ+ SPLs (5×10^5) purified by MACS were cultured in IMDM (1 mL) supplemented with 5% FBS for 24 h at 37°C. During this 24 h, PMA (50 ng/mL, Sigma–Aldrich) and ionomycin (1 μg/mL, Sigma–Aldrich) were added during the last 6 h, or an interleukin (IL)-15/IL-15R complex (50 ng/mL) was added during the entire 24-h period. IMDM with 5% FBS and streptomycin (0.1 mg/mL) was used as a positive control because the antibiotic streptomycin is known to inhibit *Escherichia coli*. After 24 h, cellular components were deposited by centrifugation, and supernatants were collected for an assay to measure antimicrobial activity.

Assay to Measure Antimicrobial Activity

Escherichia coli [a gift from Dr. Shujuan Lv (Anhui Medical University, Anhui, China)] was cultivated aerobically in liquid Luria Bertani (LB) medium at 37°C for 8 h and the concentration

was diluted to 1×10^5 CFU/mL. LB-Agar Medium petri plates were coated with *E. coli* (1×10^5 CFU/mL) and then sterile Oxford cups (8 mm in diameter) were placed on the surface of the each bacteria-inoculated agar plate. Next, supernatants (200 μL) from stimulated CD8αβ+ IELs or CD8αβ+ SPLs were dropped into the corresponding Oxford cups, and equivalent amounts of IMDM with streptomycin were used as positive controls. The plates were incubated for 18 h at 37°C. The antimicrobial activity was shown by the clear inhibition zone (called "inhibition ring") around the sample-loaded Oxford cups after incubation and the diameter of the inhibition rings were measured.

Statistical Analyses

The unpaired Student's *t*-test was used for two groups. A normal distribution was evaluated by the Kolmogorov–Smirnov test. If data showed a normal distribution, they were tested by an unpaired *t*-test (parametric test) but, if not, the Mann–Whitney test (non-parametric test) was used. A one-way ANOVA for more than two groups were used to determine significant differences. Data were presented as mean ± SEM. $P < 0.05$ was considered significant.

Data Availability Statements

Microarray data have been deposited at the National Center for Biotechnology Information GEO repository through accession number GSE105061.

RESULTS

Commensal Bacteria Specifically Regulate the Number of CD8αβ+ IELs in the Intestinal Epithelium

CD8αβ+ IELs (type A) are a population of resident effector memory T cells scattered along the epithelium of the small intestine (5). The commensal microbiota has an important role in the maintenance of immune homeostasis (31–34). Studies in GF mice have demonstrated a positive relationship between CD8αβ+ IELs and commensal bacteria within the small intestine (18–20, 31, 35), yet a systematic classification and comparison with other CD8αβ+ cells have not been made. To investigate further the crosstalk between CD8αβ+ IELs and commensal bacteria, we first verified the positive relationship between them. By flow cytometry, we gated CD45+ CD3+ CD8β+ CD8α+ TCRβ+ cells in the epithelium of the small intestine, as well as CD8αβ+ SPLs as a phenotypic comparison (**Figures 1A,B**). CD8αβ+ IELs comprised most intraepithelial T cells in SPF mice. A dramatic decrease in absolute numbers and proportions was shown in GF mice, including the proportion of CD8αβ+ IELs in all CD45+ lymphocytes and CD8+ T cells (**Figures 1C,D**). These results confirmed a role for commensal bacteria in the accumulation of CD8αβ+ IELs.

As mice age, commensal bacteria accumulate gradually in the intestine (36). The number of CD8αβ+ IELs in adult SPF mice was greater than that in newly weaned mice (**Figures 1E,F**). To mimic this condition, mice were treated with a combination of antibiotics beginning at 3 weeks of age and lasting for 5 weeks (Atb-treated mice). These mice were compared with mice that received water without antibiotics. A decrease in the number of CD8αβ+ IELs was observed in Atb-treated mice but not in mice treated with normal water. The number and proportion of CD8αβ+ SPLs were unchanged in Atb-treated mice (**Figures 1G,H**). These data demonstrated that commensal bacteria could regulate the number of CD8αβ+ IELs in the epithelium of the small intestine.

CD8αβ+ IELs Are Induced by Specific Members of Commensal Microbes

The number of CD8αβ+ IELs was affected by commensal bacteria, so the load and diversity of bacteria was analyzed in the intestine of Atb-treated mice. A sharp decrease in the load of commensal bacteria was observed after antibiotic treatment, as assessed by the difference in global microbe DNA loads between Atb-treated and water-treated mice (**Figure 2A**). This observation suggested that accumulation of CD8αβ+ IELs was influenced by the load of commensal microbes. Meanwhile, a decrease in the diversity in Atb-treated mice was shown by the Shannon index (**Figure 2B**). Moreover, the intestines of Atb-treated mice had a markedly different microbial composition, especially in the proportion of *Bacteroidetes* and *Actinobacteria* groups (**Figure 2C**). Antibiotic treatment essentially eliminated *Bacteroidetes* and *Actinobacteria*, which were predominant in mice that did not receive antibiotics (**Figure2D**). Notably, the genera of *Bacteroidetes* and *Actinobacteria* disappeared completely in Atb-treated mice, whereas the genera within other phyla (e.g., *Fimicutes* and *Tenericutes*) were partially preserved (Figure S1A in Supplementary Material). Individual antibiotic treatment also revealed that CD8αβ+ IELs were influenced by some bacteria sensitive to ampicillin (Figures S1B,C in Supplementary Material).

To elucidate whether the gut microbiota eliminated by antibiotics, such as *Bifidobacterium* in phylum *Bacteroidetes*, could induce CD8αβ+ IELs, we carried out microbiota transplantation. After initial antibiotic treatment, *Bifidobacterium* species were removed completely (**Figure 2E**). Then, *Bifidobacterium* species were transferred to these mice by gavage and, 9 days later, the appearance of *Bifidobacterium* species was confirmed by qPCR of fecal material (**Figure 2F**). Colonization by *Bifidobacterium* species induced a robust increase in the number of CD8αβ+ IELs after 15 days (**Figure 2F**). Therefore, we concluded that *Bifidobacterium* species were members of the commensal microbiota that induced accumulation of CD8αβ+ IELs in the small intestine.

TLR Signaling Is Required for Commensal Bacteria-Dependent CD8αβ+ IELs

A close relationship between commensal bacteria and CD8αβ+ IELs was demonstrated, then the regulation of CD8αβ+ IELs was explored. TLRs are expressed by macrophages, dendritic cells (DCs), and IECs, and recognize various commensal- and pathogen-associated molecular patterns (37, 38). To evaluate the impact of TLR signaling on type A IELs, CD8αβ+ IELs in various TLR-deficient mice were assessed.

The absolute number of CD8αβ+ IELs in TLR2-deficient, TLR4-deficient, and TLR9-deficient mice, and in mice deficient in all three TLRs, exhibited a similar reduction to that in Atb-treated mice. Although TLR2$^{-/-}$ TLR4$^{-/-}$ TLR9$^{-/-}$ mice showed

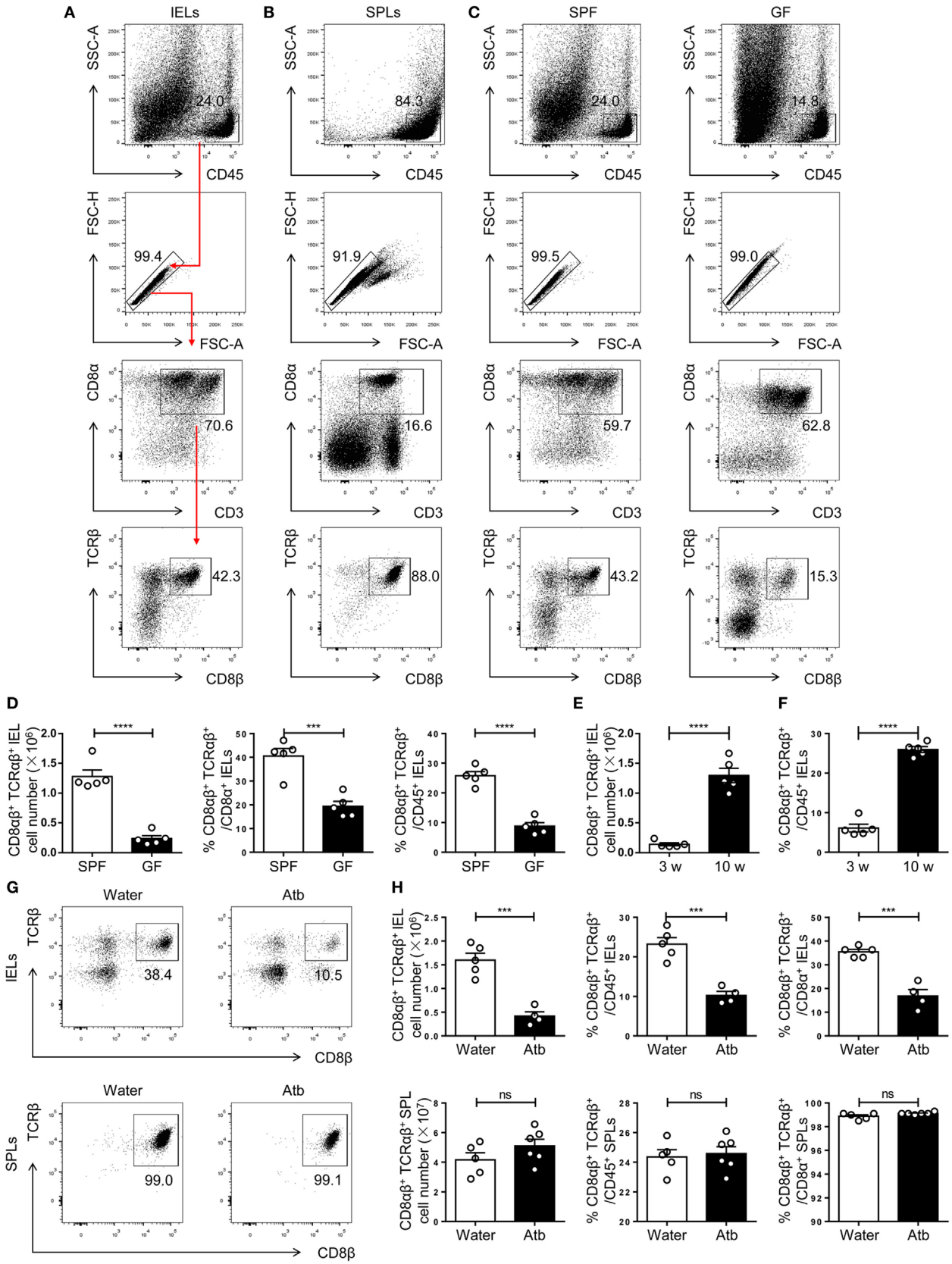

FIGURE 1 | Continued

FIGURE 1 | Commensal bacteria direct the number and frequency of CD8αβ+ IELs in the epithelium of the small intestine. **(A,B)** Gating strategy and representative flow spots of CD3+ CD8α+ CD8β+ TCRβ+ among CD45+ single cells from SPF mice. Flow cytometry was undertaken on IELs **(A)** and SPLs **(B)**. Red arrow in **(A)** indicates the order for gating. **(C)** CD8αβ+ IELs from SPF and germ-free (GF) mice on a C57BL/6 background were gated for further analyses by the strategy shown in **(A)**. **(D)** The absolute number and percentage of CD8αβ+ IELs were compared between SPF and GF mice. **(E,F)** The absolute number **(E)** and percentage **(F)** of CD8αβ+ IELs were compared between 3- and 10-week-old WT mice. **(G,H)** Three-week-old SPF mice were fed with normal water or water containing antibiotics for 5 weeks and identified, respectively, as water-treated mice and Atb-treated mice. The absolute number and percentage of CD8αβ+ IELs and SPLs from the indicated mice were analyzed by flow cytometry, $n = 4$–6 mice per group. Numbers adjacent to outlined areas denote the percentage of gated cells. All mice used were on a C57BL/6 background. Data are representative of two independent experiments. Unpaired *t*-test. Error bars represent the mean ± SEM. ***$P < 0.001$, ****$P < 0.0001$. Abbreviations: ns, not significantly different; IEL, intraepithelial lymphocyte; SPL, splenocyte; Atb, antibiotic; WT, wild-type; SPF, specific pathogen-free; GF, germ-free.

the most significant reduction in the absolute number of CD8αβ+ IELs (**Figures 3A,B**), no difference was observed in the number of CD8αβ+ SPLs in these TLR-deficient mice (**Figure 3C**). Moreover, the number of PPs and the length of small intestines showed no abnormality in TLR-deficient mice (**Figures 3D,E**). These data suggested that commensal bacteria maintained CD8αβ+ IELs indirectly *via* TLRs.

Commensal Bacteria-Dependent CD8αβ+ IELs Exhibit Potent Microbicidal Activity

Our data demonstrated an essential role for commensal bacteria in the maintenance of CD8αβ+ IELs in the epithelium of the small intestine. Hence, questions arose: why do the number of cytotoxic CD8αβ+ IELs correlate with commensal bacteria and, during homeostasis do cytotoxic CD8αβ+ IELs have other functions with regards to the commensal bacteria within the small intestine? To gain insight into these questions, CD8αβ+ IELs (purity >97%) were compared with CD8αβ+ SPLs by microarray analysis (Figure S2C in Supplementary Material). Without stimulation *in vitro*, CD8αβ+ IELs exhibited a 20-fold increase in the expression of a series of genes that mediate antibacterial processes (e.g., *Defa1*, *RegIIIγ*, and *lypd8*). At the transcriptional level, levels of the canonical effector molecule of CTLs, interferon (IFN)-γ, increased threefold in CD8αβ+ IELs relative to CD8αβ+ SPLs (Figure S2A in Supplementary Material). Expression of genes expressed specifically in Paneth cells and epithelial cells (e.g., *Sox 9*, *Ctnnb1*, *Ephb3*, *Epcam*, *Lyz 2*, *Lyz1*, and *Cd24a*) was not upregulated in CD8αβ+ IELs, demonstrating that IEL contamination by Paneth cells and epithelial cells was unlikely (Figure S2B in Supplementary Material). Among the genes whose expression was increased in CD8αβ+ IELs were those for a family of α-defensins. This included *mmp7*, a member of a class of genes encoding matrix metalloproteinases known to process inactivated mouse α-defensins to mature-active peptides (**Figure 4A**) (39). Gene ontology (GO) analyses revealed that CD8αβ+ IELs showed increased expression of the genes that mediate the defense response to Gram-negative bacteria (**Figure 4B**). RT-PCR of purified CD8αβ+ IELs and CD8αβ+ SPLs demonstrated expression of genes of the α-defensin family, including *mmp7*, in CD8αβ+ IELs (**Figure 4C**).

The presence of α-defensins in CD8αβ+ IELs was assessed by flow cytometry and immunofluorescence. Correspondingly, defensin 1 and mmp7 were detected in CD8αβ+ IELs but not in CD8αβ+ SPLs (**Figures 4D,E**). It is difficult to distinguish α-defensins in CD8αβ+ IELs from α-defensins in Paneth cells *in situ*, so purified CD8αβ+ IELs were assessed by immunofluorescence, and defensin 1-positive granules were detected (**Figure 4F**). CD8αβ+ IELs have a "resident effector memory" phenotype in that IFN-γ, granzyme B, tumor necrosis factor-α, CD44, CD69, CD103, and CD5 are detected readily (Figure S3 in Supplementary Material), as described previously (2, 5, 40). In GF- and Atb-treated mice, there was a decrease in the number of defensin 1+ CD8αβ+ IELs in comparison with untreated SPF mice, indicating that these defensin 1+ CD8αβ+ IELs were dependent upon commensal bacteria (**Figures 4G,H**). In summary, CD8αβ+ IELs contained AMPs and antimicrobial peptide-carrying CD8αβ+ IELs showed a positive relationship with commensal bacteria.

Commensal Bacteria-Dependent CD8αβ+ IELs Can Inhibit the Growth of Bacteria Directly

We had shown expression of α-defensins in CD8αβ+ IELs. Defensins are broad-spectrum AMPs that act against various bacteria (23). Hence, next we assessed the antimicrobial activity of CD8αβ+ IELs *ex vivo*. As mentioned above, Paneth cells are thought to be the only source of α-defensins in the mouse intestine. Also, the antimicrobial activity of Paneth cells by secretion of microbicidal α-defensins (also called cryptdins) was assessed by the co-culture of isolated crypt secretions on bacteria (41). Based on that study, we conducted a co-culture experiment with CD8αβ+ IELs and *E. coli*.

Sorted CD8αβ+ IELs and CD8αβ+ SPLs were cultured for 24 h, and activated with PMA and ionomycin for the final 6 h, after which supernatants were collected, and antimicrobial activity determined. As described in Section "Materials and Methods," petri plates containing LB-Agar medium were coated with *E. coli* and then Oxford cups were placed on the plates and filled with supernatants. The petri plates were transferred into an incubator at 37°C, and bacterial colonies and inhibition rings were observed 18 h later. No inhibition rings were observed around Oxford cups filled with supernatants from purified CD8αβ+ SPLs, but inhibition rings were observed around Oxford cups filled with supernatants from purified CD8αβ+ IELs (Figure S2D in Supplementary Material; **Figure 5A,B**). These data showed that secretion from stimulated CD8αβ+ IELs could inhibit the growth of bacteria, likely due to AMPs such as defensins.

Interleukin-15 has been reported to influence the activation and proliferation of small-intestinal TCRγδ+ IELs as well as peripheral CD8+ T cells (42–46). Hence, the effect of IL-15 on the secretion of AMPs by CD8αβ+ IELs was assessed. *E. coli* was cultured with IELs

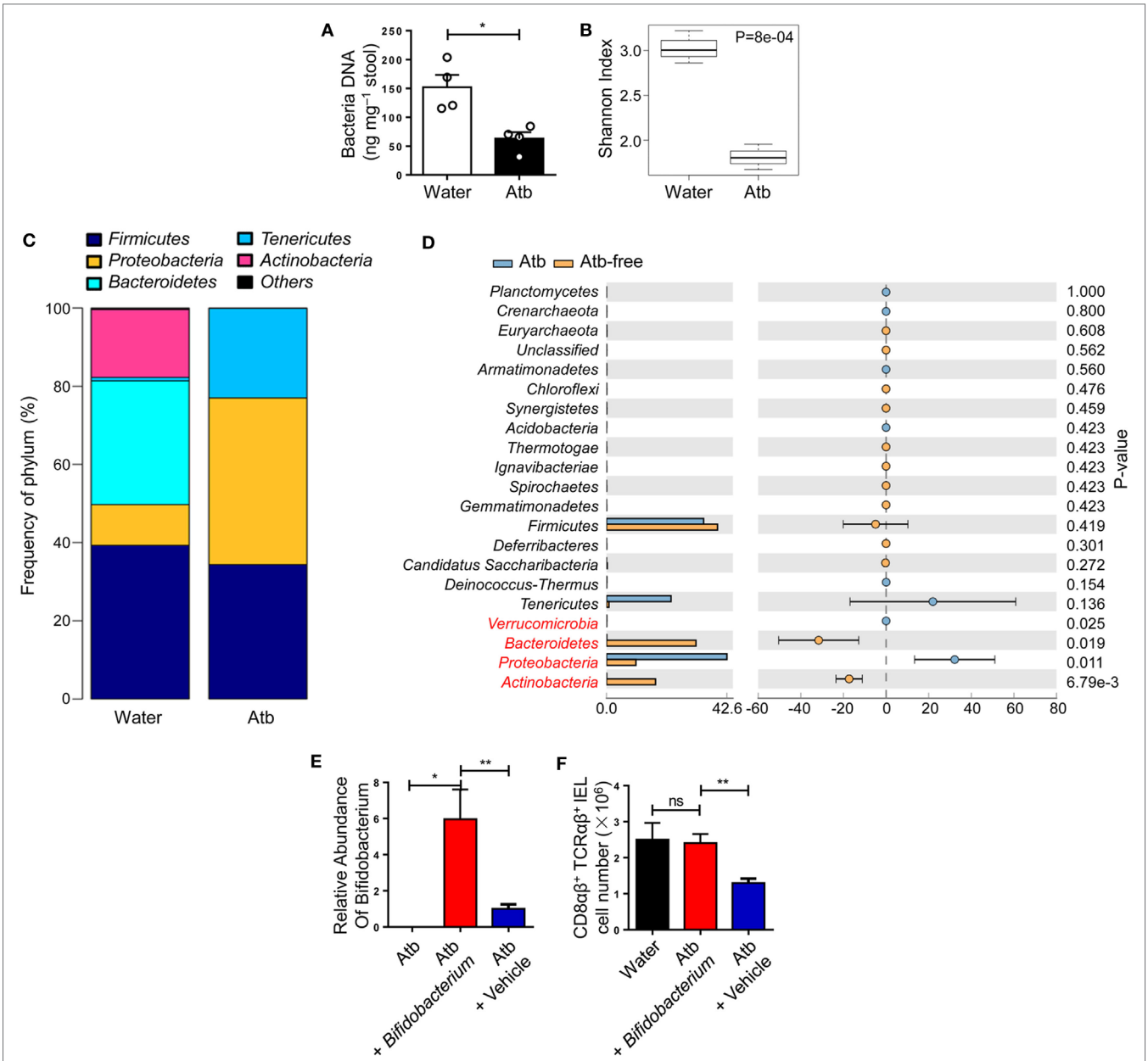

FIGURE 2 | Global diversity of commensal bacteria affects the quantity of CD8αβ+ IELs. **(A–D)** Three-week-old mice were treated with normal water or water containing a combination of antibiotics (metronidazole, neomycin, ampicillin, and vancomycin) for 5 weeks. Analyses of 16S rDNA were done on bacterial DNA in the contents of the distal small intestine. **(A)** DNA load of bacteria in feces were detected (2 weeks post-treatment). **(B)** Plot of diversity using the Shannon Index. The Shannon Index of the two groups is shown to indicate the diversity of species. **(C)** Relative percentages of the dominant phylum in water-treated and Atb-treated mice were evaluated by the mean reads. **(D)** Distribution of intestinal bacterial groups in water-treated versus Atb-treated mice. The left portion indicates the proportion of the variety of bacteria phyla in water-treated and Atb-treated mice. The right portion indicates the difference in mean proportions between Atb-treated and water-treated groups, along with *P*-values and confidence intervals. **(E)** Quantitative PCR of bacterial DNA from fresh stool samples of mice pretreated with antibiotics for 2 weeks (left column); Atb-pretreated mice administered (i.g.) with *Bifidobacterium* (middle column) or vehicle (right column) (9 days post-transfer). Data show the relative abundance of *Bifidobacterium* in each group. **(F)** The absolute number of CD8αβ+ TCRαβ+ IELs was detected (15 days post-transfer). Data from mice treated with normal water from birth (left column), Atb-pretreated mice administered (i.g.) with *Bifidobacterium* (middle column), or vehicle (right column) (15 days post-transfer) are shown. Data are representative of two independent experiments. Unpaired *t*-test. Error bars represent the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviations: ns, not significantly different; Atb, antibiotic; IEL, intraepithelial lymphocyte.

and bacterial growth was judged by the color of the culture medium. Culture medium with IELs and 10^4 CFU of *E. coli* turned yellow after 24 h, whereas the culture medium with IELs and 10^4 CFU of *E. coli* with added IL-15 did not turn yellow (**Figure 5C**). According to the antimicrobial assay described above, supernatants from purified CD8αβ+ IELs stimulated with IL-15 also produced inhibition

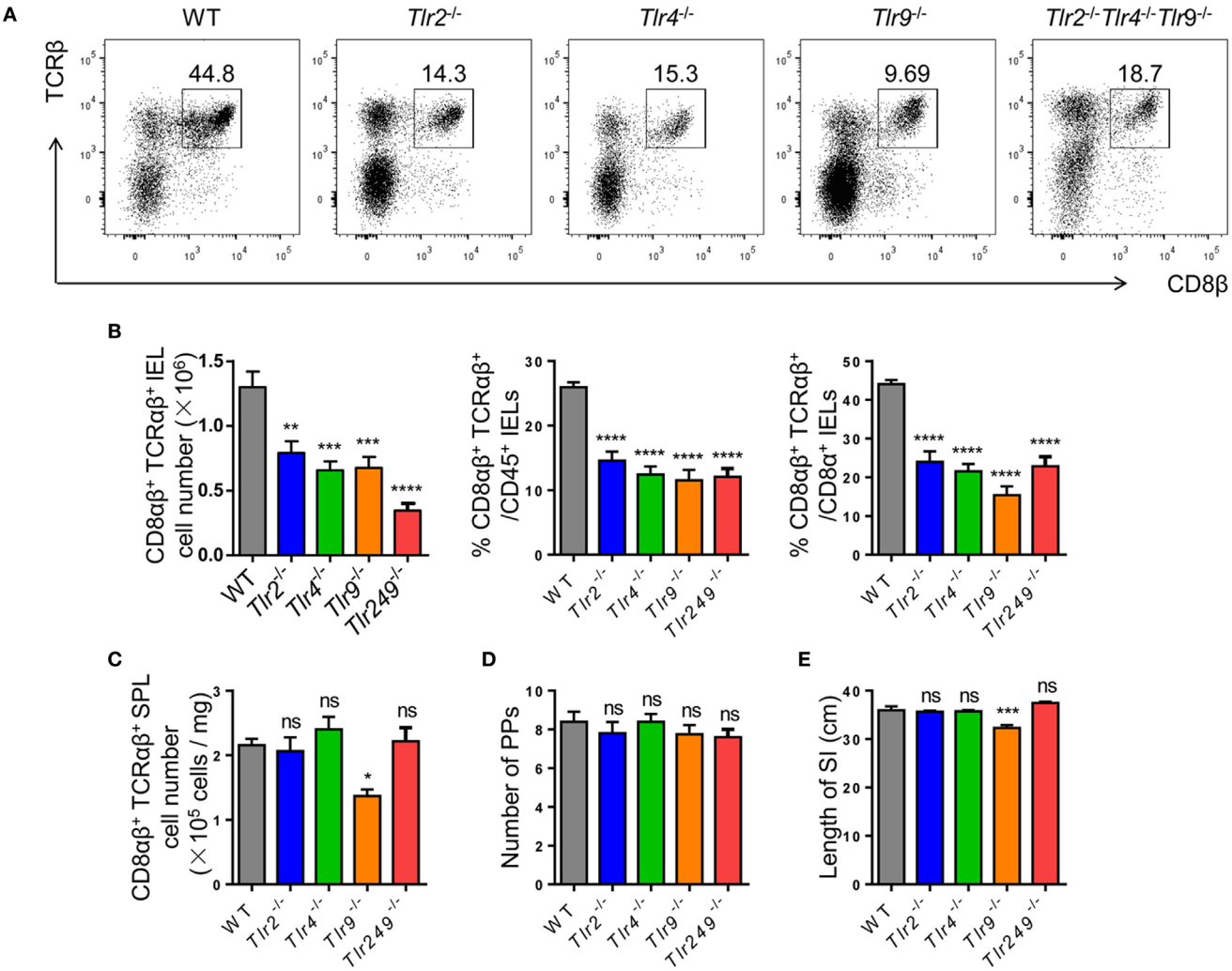

FIGURE 3 | Commensal bacteria regulate the number of CD8αβ+ IELs *via* TLR (TLRs). **(A,B)** CD8αβ+ IELs from WT, TLR2−/−, TLR4−/−, TLR9−/−, and TLR2−/−TLR4−/−TLR9−/− mice were gated by the strategy shown in **Figure 1A (A)**. Absolute number and percentage of CD8αβ+ IELs from indicated mice were calculated **(B)**. **(C–E)** The absolute number of CD8αβ+ splenocytes **(C)**, number of PP's scattered along the small intestine **(D)**, and length of the entire small intestine **(E)** in indicated mice were counted and compared, $n = 4–5$ mice per group. All mice used were on a C57BL/6 background. Data are representative of three independent experiments. One-way ANOVA followed by Dunnett's test. Error bars represent the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Abbreviations: ns, not significantly different; IEL, intraepithelial lymphocyte; SPL, splenocyte; PPs, Peyer's patches.

rings (**Figures 5D,E**). These data suggested that IL-15 promoted the antimicrobial activity of CD8αβ+ IELs.

DISCUSSION

The intestinal epithelium serves as a primary barrier between the host and a large number of bacteria in the lumen of the small intestine. A large proportion of IELs at this site are CD8αβ+. However, as CD8αβ+ IELs are closely correlated with commensal bacteria, their function besides cytotoxicity is unclear. Nor is their role in homeostasis established. Herein, commensal bacteria were demonstrated to regulate the number of CD8αβ+ IELs along the intestinal epithelium. Also, the importance of TLRs in this process was shown. Microarray analysis identified a series of genes that encode antibacterial peptides in commensal-dependent CD8αβ+ IELs. The supernatants of these IELs were found to have direct antibacterial activity. These results suggest that commensal-dependent CD8αβ+ IELs can inhibit the growth of bacteria effectively.

MyD88 signaling has been reported to induce IL-15 production from IECs and, in addition, to induce the accumulation of TCRγδ+CD8αα+ and TCRαβ+CD8αα+ IELs (42). CD8αβ+ IELs are located in the same microenvironment, so they may also be regulated by IL-15 induced *via* TLR-Myd88 signaling. CD8αβ+ IELs do not express TLRs, so it is possible that a cytokine (e.g., IL-15) may mediate the effect of commensal bacteria on CD8αβ+ IELs, because lower expression of IL-15 in GF mice in comparison with SPF mice was observed (data not shown). IECs, intestinal macrophages, and DCs express IL-15, and macrophages from gut microbiota-depleted mice show lower expression of

IL-15 (47). We demonstrated that the IL-15/IL-15R complex can enhance the antimicrobial activity of CD8αβ⁺ IELs *in vitro* (**Figures 5D,E**). These data suggested that IL-15 expression was influenced by commensal bacteria, and raised the possibility that IL-15 may mediate the effect between commensal bacteria and CD8αβ⁺ IELs.

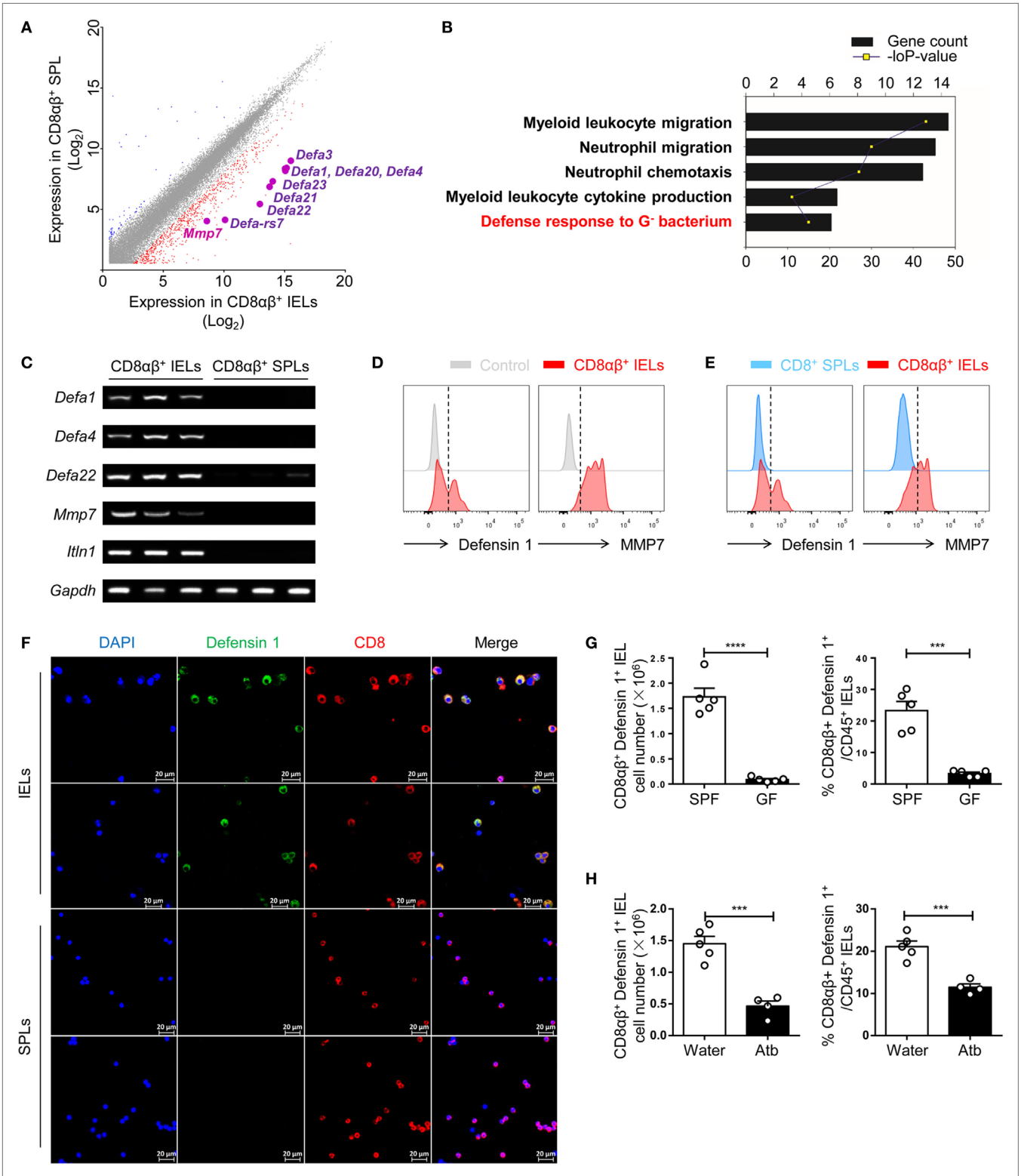

FIGURE 4 | Continued

FIGURE 4 | Expression of a series of genes that mediate the microbicidal response is upregulated in CD8αβ+ IELs. **(A)** CD45+ CD3+ CD8α+ CD8β+ TCRβ+ single cells were sorted from IELs and SPLs and subjected to microarray analysis. The difference in gene expression by microarray analysis is shown in a scatterplot and compares CD8αβ+ IELs and CD8αβ+ SPLs. Expression of several genes encoding α-defensins was upregulated in CD8αβ+ IELs and are highlighted. Two samples were analyzed per group with each sample sorted from 10 wild-type mice. **(B)** Gene ontology (GO) analysis of the genes upregulated in CD8αβ+ IELs. Five representative pathways were selected from the top 30 from GO enrichment. **(C)** The mRNA expression of *Defa1*, *Defa4*, *Defa22*, *Mmp7*, and *Itln1* in CD8αβ+ IELs and CD8αβ+ SPLs was measured by RT-PCR. Three samples were analyzed per group with each sample sorted from 10 wild-type mice. **(D,E)** Expression of defensin 1 and MMP7 in CD8αβ+ IELs (red histogram), control staining (gray histogram), and CD8αβ+ SPLs (blue histogram) detected by flow cytometry in gated CD45+CD3+CD8α+ CD8β+TCRβ+ single cells. The control in **(D)** (gray histogram) denoted that no primary antibody but only fluorescent secondary antibody had been added. Data are representative of three independent experiments. **(F)** IELs and purified CD8αβ+ SPLs were collected and the presence of defensin 1 (green) in CD8+ (red) IELs and SPLs detected by immunofluorescence. Data are representative of two independent experiments. **(G,H)** The absolute number and proportion of defensin 1+CD8αβ+ IELs were calculated and compared between GF and SPF mice **(G)** or water-treated mice and Atb-treated mice **(H)**, n = 4–5 mice per group. GF and SPF mice on a BALB/c background were used in **(G)**, but all the other mice used in this figure were on a C57BL/6 background. Data are representative of two independent experiments. Unpaired t-test. Error bars represent the mean ± SEM. ***$P < 0.001$, ****$P < 0.0001$. Abbreviations: IEL, intraepithelial lymphocyte; SPL, splenocyte; SPF, specific pathogen-free; GF, germ-free; Atb, antibiotic.

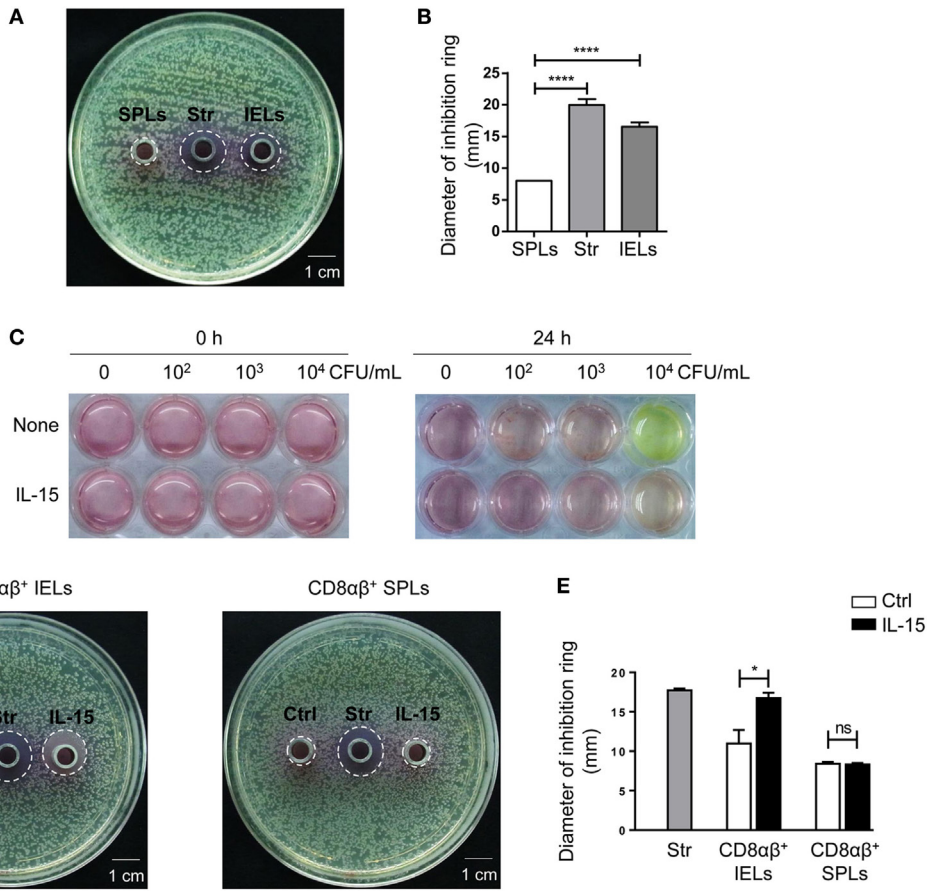

FIGURE 5 | The supernatant from CD8αβ+ IELs can inhibit the formation of bacterial colonies directly. **(A,B)** CD8αβ+ IELs and CD8αβ+ SPLs were sorted and cultured for 24 h, with activation by PMA and ionomycin during the final 6 h. After 24 h, the supernatants were collected for an antimicrobial-activity assay. Iscove's modified Dulbecco's medium (IMDM) with streptomycin (0.1 mg/mL) was added to the middle Oxford cup as a positive control (labeled as "Str," middle). Supernatants from CD8αβ+ SPLs (labeled as "SPLs," left) and CD8αβ+ IELs (labeled as "IELs," right) were added into Oxford cups, respectively, on the left and right side of the agar plate **(A)**. Inhibition rings were observed after 18 h. The diameter of the inhibition rings was measured and is shown in **(B)**. One-way ANOVA followed by Dunnett's test. Each cell sample was sorted from 5 wild-type mice. Data are representative of three independent experiments; error bars represent the mean ± SEM. ****$P < 0.0001$. **(C)** IELs (1×10^6 cells) and increasing concentrations of *Escherichia coli* (CFU/mL), indicated in the Figure, were co-cultured with or without IL-15. The color of the medium indicated the acidity produced by bacterial proliferation. Data are representative of three independent experiments. **(D,E)** CD8αβ+ IELs were sorted and cultured with or without the IL-15/IL-15R complex for 24 h, after which the supernatants were collected for an antimicrobial-activity assay. IMDM with streptomycin (0.1 mg/mL) was added to the middle Oxford cup as a positive control (labeled as "Str," middle). Oxford cups containing supernatants from CD8αβ+ IELs activated without (labeled as "Ctrl," left) or with (labeled as "IL-15," right) the IL-15/IL-15R complex were located, respectively, on the left and right side of the agar plate **(D)**. Inhibition rings were observed after 18 h. The diameter of the inhibition rings was measured and is shown in **(E)**. Unpaired t-test. Each cell sample was sorted from 5 wild-type mice. Data are representative of two independent experiments. Error bars represent the mean ± SEM. *$P < 0.05$. Abbreviations: ns, not significantly different; IEL, intraepithelial lymphocyte; SPL, splenocyte; Str, streptomycin.

Analyses of the 16S rDNA of Atb-free mice and Atb-treated mice in this study suggested that CD8αβ+ IELs may be influenced by specific groups of bacteria. It was reported recently that *Lactobacillus reuteri*, a probiotic bacterium whose numbers were decreased dramatically by the antibiotics in their study, induced CD4+CD8αα+ T cells in the intestinal epithelium (48). That recent report raised the possibility that particular bacterial species may induce specific subsets of IELs because they are located in a similar microenvironment. Our microbiota-transplantation experiment showed that *Bifidobacterium* species, removed by antibiotics in our study, could induce an increase in the number of CD8αβ+ IELs effectively. How these bacteria influence CD8αβ+ IELs or other IEL subsets at the mechanistic level is not known.

Comparative microarray analysis of CD8αβ+ IELs and CD8αβ+ SPLs cells showed that expression of a series of genes encoding AMPs (especially α-defensins) was upregulated in CD8αβ+ IELs but not SPLs. Studies have suggested that Paneth cells are the only source of α-defensins in mice (21–23). CD8αβ+ T cells in long-term HIV-1 non-progressors were demonstrated to secrete α-defensin 1, 2, and 3 and suppress HIV-1 replication. In that study, CD8αβ+ T cells did not express mRNA for the defensins but rather acquired α-defensin protein by taking up defensins secreted originally by neutrophils (49–51). It has also been reported that, in some cases, CD8+ T cells among peripheral-blood lymphocytes from patients with severe cutaneous reactions can upregulate expression of α-defensin 1–3, and that this correlates with disease severity (52). These findings suggest that the function of CD8+ T cells can be expanded under specific physiologic conditions. In our study, a series of α-defensins were found specifically in CD8αβ+ IELs at gene and protein levels in homeostasis. Furthermore, supernatants from purified CD8αβ+ IELs inhibited the growth of bacteria. Taken together, these data demonstrate murine CD8αβ+ IELs to be producers of α-defensins in the intestine.

α-defensins are broad-spectrum microbicides that are major determinants of intestinal micro-ecology (53). For many years, Paneth cells were thought to be the only source of α-defensins and, as such, were considered important in host–microorganism homeostasis. Paneth cells are located at the base of crypts between villi. As a result, secreted bactericidal peptides are released into the lumen from the base of the crypts, with maximal antimicrobial activity at the bottom of the crypt, but a decreased concentration and activity in the middle and at the top of the villi. In this study, CD8αβ+ IELs distributed along the epithelial layer of the small intestine were demonstrated to secrete α-defensins, which may be important as supplements to the α-defensins produced by Paneth cells. Together, CD8αβ+ IELs and Paneth cells may combine effectively to prevent bacterial invasion. This hypothesis is consistent with the concept that CD8αβ+ IELs and the microbiota regulate each other reciprocally.

Interestingly, investigation of another subset of IELs in the intestinal epithelium, TCRγδ+ IELs, revealed that the latter could secrete RegIIIγ and RegIIIβ, which are antimicrobial factors of the small intestine known to protect against invading resident bacteria (54). Taken together with the data herein, those findings suggest that the intestinal epithelium is a specialized microenvironment that serves as a physical and immune barrier to bacteria at the mucosal interface. Lymphocytes at this surface may help control pathogenic and resident bacteria to maintain host–microbial homeostasis. It is possible that the production of AMPs could also be found in T cells located in other mucosal tissues.

There may be some key factors in this microenvironment that induce the production of defensins and other AMPs by IELs. The data herein suggest that IL-15 may be a candidate, and this issue needs more investigation. In conclusion, this study demonstrated a close relationship between commensal bacteria and CD8αβ+ IELs. It uncovered a new function for CD8αβ+ IELs in the intestinal epithelium, and implies that lymphocytes in certain special microenvironments may gain new functions. Exploration of these cells in differing microenvironments may provide new insights into treatment for bacterial-based diseases and disorders.

ETHICS STATEMENT

All of the animal protocols were approved by Local Ethics Committee for Animal Care and Use at University of Science and Technology of China (USTCACUC1601007). The sample size was determined by the "resource equation" method.

AUTHOR CONTRIBUTIONS

BC designed, carried out and analyzed the experiments, and wrote the manuscript. XN analyzed part of the data. RS established methods for flow cytometry, supervised the experiments, and revised the manuscript. BZ and HongW provided GF mice. ZT and HaimingW provided the study strategy, supervised research, and revised the 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/fimmu.2018.01065/full#supplementary-material.

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Microbiome and Allergic Diseases

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Allergic diseases, such as respiratory, cutaneous, and food allergy, have dramatically increased in prevalence over the last few decades. Recent research points to a central role of the microbiome, which is highly influenced by multiple environmental and dietary factors. It is well established that the microbiome can modulate the immune response, from cellular development to organ and tissue formation exerting its effects through multiple interactions with both the innate and acquired branches of the immune system. It has been described at some extent changes in environment and nutrition produce dysbiosis in the gut but also in the skin, and lung microbiome, inducing qualitative and quantitative changes in composition and metabolic activity. Here, we review the potential role of the skin, respiratory, and gastrointestinal tract (GIT) microbiomes in allergic diseases. In the GIT, the microbiome has been proven to be important in developing either effector or tolerant responses to different antigens by balancing the activities of Th1 and Th2 cells. In the lung, the microbiome may play a role in driving asthma endotype polarization, by adjusting the balance between Th2 and Th17 patterns. Bacterial dysbiosis is associated with chronic inflammatory disorders of the skin, such as atopic dermatitis and psoriasis. Thus, the microbiome can be considered a therapeutical target for treating inflammatory diseases, such as allergy. Despite some limitations, interventions with probiotics, prebiotics, and/or synbiotics seem promising for the development of a preventive therapy by restoring altered microbiome functionality, or as an adjuvant in specific immunotherapy.

Keywords: microbiome, microbiota, allergy, allergic diseases, prebiotics, probiotics, synbiotics

INTRODUCTION

Allergic diseases, include heterogeneous inflammatory pathologies such as respiratory and food allergies (FA), which are characterized by an immunological response with T lymphocytes producing IL-4, IL-5, and IL-13 and low production of IFN-γ (Th2) (1) and others producing IL-9 and IL-10 (Th9) (2) as the main effector T cells. They promote the induction of other effector cells involved in allergic inflammation, such as mast cells, basophils, and eosinophils (1). These diseases have dramatically increased in prevalence over the last few decades (3–6) and recent research points to a central role of the microbiota (7, 8). It is well established that the microbiome can modulate the immune

response, from cellular development to organ and tissue formation (9) exerting its effects through multiple interactions with both the innate and acquired branches of the immune system. In the late 80s, Dr. Strachan proposed what is now referred to as the "hygiene-hypothesis" (10), in which changes in environment and nutrition produce a dysbiosis in the skin, gut, or lung microbiome inducing qualitative and quantitative changes in composition and metabolic activity (11, 12). Furthermore, it was proposed that a lower incidence of infection in early childhood, which may be associated with low microbiota diversity, could explain the increase in prevalence of atopic diseases (13). It should be pointed out that the hygiene hypothesis has not been found to apply to individual hygiene [no relation between personal or home cleanliness and increased risk of asthma or allergy has been found (14)], but to independent host factors such as number of older siblings, contact with pets and rural versus urban living, all of which have been shown to affect microbiome composition and the development of immunologic tolerance (15). Today, the use of bacterial culture-independent tools such as next-generation sequencing to identify different microbes has permitted the investigation of complex populations and their roles in health and disease. Here, we review the potential role of the skin, respiratory, and gastrointestinal tract (GIT) microbiomes in allergic diseases.

MICROBIOME

The term "microbiome" refers to the microorganisms that live on or inside another organism. They interact with each other and with their host and can be classified as beneficial (symbiotic) or dangerous (pathogenic) (16). Microbiome in humans can account for 90% of the cells by a ratio of 10:1 (17). New studies point out that the number of bacteria in the body is of the same order as the number of human cells (18). Most of these microorganisms inhabit the gut. The microbiome effectively adds a huge amount of genes to the human genome, potentially increasing it up to 200 times (19). As a result, the composition of the human microbiome could be important in the context of health or disease.

Human Gut Microbiome and Implications in Food Allergy

The GIT has a very important immune function in developing either effector or tolerant responses to different antigens by balancing the activities of Th1 and Th2 cells as well as regulating Th17 and T regulatory (Treg) cells in the lamina propria (20–23). Immune dysfunction in allergic diseases such as asthma and atopy seems to be related to differences in the function and composition of the gut microbiome (24).

The gut microbiome constitutes a highly complex ecosystem which includes eukaryotic fungi, viruses, and some archaea, although bacteria are the most prominent components (25). Its composition is generally formed during the first 3 years of life (26); however, recent work has suggested that its colonization may begin *in utero* (27), contrary to the widely held dogma of the fetus as a sterile environment. Despite its early formation, its composition is highly dynamic and dependent on host-associated factors such as age, diet, and environmental conditions (26, 28–31) with the major phyla being *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*. The gut microbiome is not homogeneous throughout the GIT, showing higher diversity in the oral cavity and intestine, and lower diversity in the stomach, mainly because of the acid environment (32). Aerobic species are mainly located in the upper small intestine and anaerobic species in the colon (33).

Most antigens in the GIT come from dietary factors and gut microbiota, both of which can affect immune tolerance being the promotion of Treg cells to these dietary factors crucial to avoid an immune response to dietary antigens (34). Alterations in GIT bacterial levels or diversity (dysbiosis) can disrupt mucosal immunological tolerance, leading to allergic diseases including FA (35) and even asthma (36–38). Moreover, low IgA levels at the intestinal surface barrier can also contribute to FA. In fact, low microbiota levels and IgA appear to be related: gut microbiota can stimulate dendritic cells (DCs) in the Peyer's patches (digestive type of mucosa lymphoid-associated tissue) to activate B cells, leading to specific IgA antibodies production through class switching (39). This stimulation may occur through the production by members of the microbiome of metabolites, such as short chain fatty acids (SCFAs). Thus, the immune tolerance network in the intestinal lumen can be considered to include the gut microbiota, their metabolic products, dietary factors, epithelial cells, DCs, IgA antibodies, and regulatory T cells (**Figure 1**).

Several factors associated with dysbiosis may influence FA, such as cesarean versus vaginal delivery (40), low versus rich fiber diet (41), breastfeeding (42), and/or early-life-antibiotic exposure, all of which affect bacterial load and diversity.

Once thought to be almost sterile, the esophagus has been shown to comprise around 300 bacteria species. Significant differences in the microbial composition of children with active esophageal inflammation caused by eosinophilic esophagitis compared with controls have been reported (43). Importantly, both the degree of inflammation and the treatment regimen seem to impact the esophageal microbiota (43).

Human Lung Microbiome and Implications in Respiratory Allergy

As with the esophagus and fetus, the lung has long been thought of as sterile; however, recent evidence has shown it to harbor various bacteria phyla, including *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*, even in healthy subjects (44). Similar to the gut, the lung microbiome changes rapidly in the first years of life, before beginning to stabilize (45, 46). Colonization occurs gradually in healthy children, starting with *Staphylococcus* or *Corynebacterium*, followed by *Moraxella* or *Alloiococcus* (46). A breakdown in the development of the commensal population can lead to dysregulation of the IgE–basophil axis, with elevated serum IgE concentrations and increased of circulating basophil populations as has been described in murine models of allergic airway disease (47). Importantly, this link was found to be B-cell intrinsic and dependent on the MYD88 pathway. Moreover, the lung microbiome may also play a role in driving asthma endotype polarization, by adjusting the balance between Th2 and Th17 patterns. *Enterococcus faecalis* can suppress Th17 immunity and symptoms of allergic airway disease, and thus it has even been considered a potential therapeutic agent for both asthma and Th17 immunity (48).

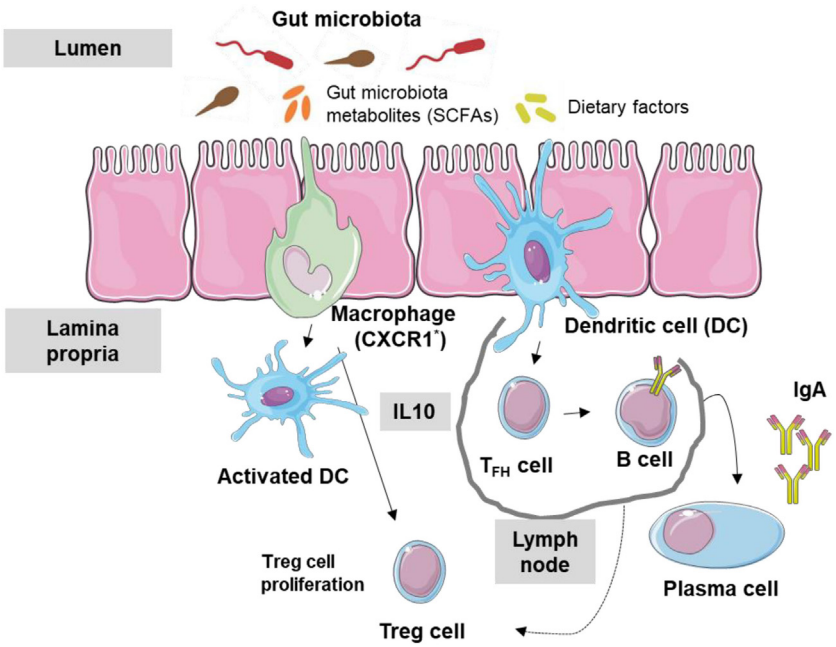

FIGURE 1 | Interaction between gut microbiota and immune system. Gut microbiota metabolites and dietary factors constitute the main antigen load of the gastrointestinal tract. Macrophages (CXCR1+) and dendritic cells (DCs) are stimulated and T regulatory (Treg) cells are activated by metabolic products such as short chain fatty acid (SCFA). Follicular T cells activate B cells inducing the production of IgA antibodies.

Differences in levels and diversity of the lung microbiome have been found between healthy people and patients with asthma and allergic diseases, with an increase of Proteobacteria in the latter; moreover, their presence has been linked to increased severity of asthma probably through the upregulation of Th17-related genes (49, 50).

Early colonization with *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus pneumoniae* has been associated with recurrent wheezing and asthma (45, 46, 51, 52). Importantly, as well as bacteria, viruses will also influence asthma development, as has been demonstrated with human rhinovirus infections of the nasopharynx in early-life (46). In addition, other associations such as helminths may be protective for asthma, as helminth infections have been shown to increase the microbiota diversity (53). Associations have been found between the composition of the lung and gut microbiome and the risk of respiratory allergic disease development (54) indicating that both gut and lung mucosa may function as a single organ, sharing immunological functions (44).

Skin Microbiome and Cutaneous Allergic Diseases

Bacterial dysbiosis is associated with chronic inflammatory disorders of the skin, such as atopic dermatitis (AD) and psoriasis (55). The composition of the skin microbiota depends on the body site samples (56). The relevance of AD, often associated with other allergic diseases, has significantly increased in the last few decades. Outgrowths of *Staphylococcus* and reductions of other communities like *Streptococcus* or *Propionibacterium* species correlate with AD flares (57). On the other hand, skin commensal *Acinetobacter* species have been reported to protect against allergic sensitization and inflammation, playing an important role in tuning the balance of Th1, Th2, and anti-inflammatory responses to environmental allergens (58). Interestingly, studies of cutaneous allergic diseases have found an association with gut microbiome dysbiosis (59), although the underlying mechanisms are still unclear. An initial study of 90 patients with established AD found enrichment for *Faecalibacterium prausnitzii* and decreased levels of SCFAs in the gut (60).

Therefore, we can summarize that changes in environment and diet produce dysbiosis in gut, skin, and/or lung microbiome inducing qualitative and quantitative changes in the microbiota which directly affect the immunological mechanisms implicated in the prevention of allergic diseases (**Figure 2**).

FACTORS AFFECTING MICROBIOME DIVERSITY

Childbirth

The mode of delivery in childbirth can produce profound differences in the infant gut microbiome, with lower level of *Escherichia coli*, *Bifidobacterium*, and *Bacteroides* species in children born through cesarean section compared with those delivered vaginally (28, 61, 62). Cesarean-born infants typically have a microbiome enriched with *Staphylococcus* and *Streptococcus*, comparable with the maternal skin microbiome (63). These differences appear to be associated with higher risk of allergic diseases and asthma (64–66). Transfer of maternal vaginal microbes at birth may

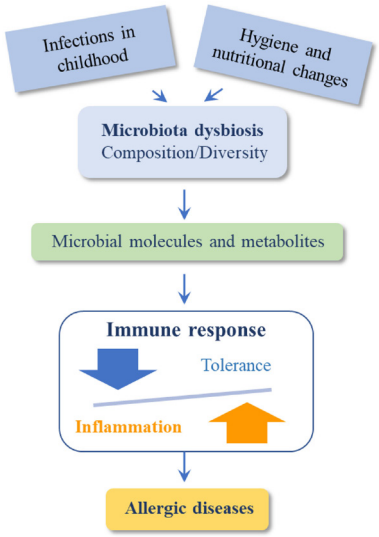

FIGURE 2 | Dysbiosis induce qualitative and quantitative changes in the microbiota that directly affect immunological mechanisms leading to allergic diseases.

mitigate these effects (67). Time of gestation may also be a factor: premature births are associated with alterations of the gut microbiome, but not atopic sensitization (68).

Importance of Early-Life Microbiome

There is mounting evidence that early-life exposure is critical for the microbiome and that gut microbial dysbiosis heavily influences immune system development (53). Potential factors include perinatal exposure to maternal or infant diet, antibiotic use, and contact with older siblings (16). Data from different populations show that the highest interindividual microbial variability occurs during the first 3 years of age (26). Noteworthy, contact with the microbiome can start before birth, since a low-abundance microbiota in the placenta (69) and meconium (70, 71) have been found.

Microbial exposure during the first months of life induces the activation of the innate immune system in different ways, with consequences for FA. Early inoculation with spore-forming *Clostridium* class IV and XIV species (72) and other bacteria (53) leads to decreased levels of circulating IgE in adulthood. Conversely, 3-week-old neonates with a higher fecal burden of *Clostridium difficile* and a higher ratio of *C. difficile* to *Bifidobacterium* showed increased numbers of skin test positive results to food and aero-allergens (73). Similarly, high levels of fecal *E. coli* in infants during their first month are associated with IgE-mediated eczema (74, 75).

Remarkably, the same colonization pattern can have different consequences at different ages. For example, colonization of *S. pneumoniae*, *H. influenzae*, or *M. catarrhalis* within the first month of life increases the risk of asthma, leading to high counts of atopic markers such as eosinophils and serum IgE, but not when colonization occurs at 12 months (45).

Furthermore, respiratory tract infections during early-life are associated with asthma development (76, 77). This may be because viral infections favor other opportunistic respiratory pathogens such as *M. catarrhalis* and *S. pneumoniae*, increasing the risk of asthma exacerbations (78). Other possible mechanisms may involve respiratory rhinovirus interacting with airway epithelial cells, increasing IL-25 and IL-33 production and contributing to Th2 immune responses (79). This is in line with the higher levels of house dust mite-specific IgE found in children infected with rhinovirus (80). Moreover, rhinovirus infection can also induce mucus hypersecretion and airway hyperresponsiveness in neonatal mice compared with adults (81).

Diet and Microbiome Metabolic Products

Another key factor influencing gut microbiome diversity is infant feeding, and especially breastfeeding, which has been shown to increase colonization by *Lactobacilli* and *Bifidobacteria* (82). Breast milk contains oligosaccharides and a wide range of fatty acids, which will affect the gut microbiome and its capacity to produce metabolites that protect against allergies and asthma (83) through the development of Treg cells (84). This effect is also produced by the intake of unprocessed milk during the first year of life, probably related to higher levels of peptides in the serum fraction and unsaturated omega-3 fatty acids (85). Other dietary components such as polyphenols and fish oils are also important for microbiome diversity (86–88).

Some noteworthy bacteria, such as *Lachnospiraceae* and *Ruminococcaceae*, can also influence the gut microbiome by producing SCFAs—including propionate, butyrate, and acetate—through fermentation of complex dietary carbohydrates. Importantly, besides acting as an essential energy source for gastrointestinal colonocytes, these acids exert various anti-inflammatory effects on the immune system that can modulate FA and respiratory diseases (89, 90), by increasing epithelial barrier function (91), and inducing Treg cells (colonic $CD103^{+}FoxP3^{+}$ cells), DCs precursors, and IL-10 production (8, 90).

Importance of Exposure to Antibiotics

The introduction of antibiotics in the 1950s is associated with an increasing incidence of allergy. This is thought to be causes by antibiotics inducing dysbiosis which has been shown to directly impact the development of AD (92) and asthma (48). The age of initial exposure could be important since maternal intake of antibiotic during pregnancy increases the risk of allergy in children (93), and antibiotic use in the first month of life has been associated with cow's milk allergy (94). Intrapartum antibiotics have been shown to lead to a modified microbiome in children at 3 and 12 months (95). Other studies showed that antibiotics affect the microbiome in older subjects (96, 97). Antibiotic administration is associated with severe allergic airway inflammation in neonates, but not in adults (98).

Even low doses of antibiotics can affect microbiome composition (99); however, the associations between antibiotic consumption and allergic diseases increase with the number of antibiotics prescribed, and variable effects have been found for different antibiotic families. Some studies have indicated

that betalactam antibiotics are the most common triggers when FA is diagnosed before 2 years of age, while macrolides are associated with FA when it is diagnosed later (100). For asthma, further studies are needed to clarify whether it is the infection rather than the antibiotics themselves that increase susceptibility (101).

INTERVENTIONS

The microbiota can be considered a therapeutical target for treating allergy; moreover, certain species can be used to enhance tolerance response induction. Different approaches for restoring the microbiome involve probiotics, prebiotics, and synbiotics.

Probiotics

According to the Food and Agriculture Organization of the United Nations and the World Health Organization, probiotics are defined as "*live microorganisms which, when administered in adequate amounts, confer a health benefit to the host*" (102). They do so by promoting the appropriate balance of gut microbiota. The health benefits attributed to one probiotic strain are not necessarily applicable to another one even within one given species (103). Furthermore, the effectiveness may depend on the time of intervention and aspects of the current microbiota composition. In fact, different studies have shown that timing is crucial (104).

In the case of FA, co-administration of bacterial adjuvants with oral immunotherapy (OIT) has been suggested as a potential treatment. Probiotic therapy with *Lactobacillus rhamnosus* increases efficacy when co-administered with peanut OIT—producing desensitization in 82% of treated patients (105)—or with hydrolyzed casein in milk allergic patients, in which an increase of fecal butyrate levels were found (106, 107). However, other strains of *Lactobacilli* and/or *Bifidobacteria* did not demonstrate any effect in preventing allergic diseases (106, 107). Some investigations have shown that the oral administration of probiotics may benefit allergic rhinitis patients (108–110); similarly, local nasal administration of *Lactococcus lactis* NZ9000 can affect local and systemic immune responses against *S. pneumoniae* (111). However, Ivory et al. reported that even oral delivery of *Lactobacillus casei* Shirota modified the immune system of allergic individuals (110), these modifications did not have a significant impact on the allergic status (112), highlighting the fact that analysis of immune parameters *per se* is not a real indicator of the therapeutical properties of the probiotics.

It has been suggested that probiotics can help preventing eczema and they also show some beneficial effects for other allergic diseases including asthma (113–117); furthermore, another approach based on the intranasal application of bacterial products (endotoxin or flagellin) has demonstrated immunomodulatory ability, mimicking the effect of probiotics, for the lung in different animal models, reducing experimental asthma by either re-establishing the expression of the ubiquitin-modifying enzyme A20 at the endothelial barrier or inducing Tregs (118, 119).

Therefore, it seems that the optimal time periods to apply probiotic intervention are before, during, and just after birth represents. Nevertheless, more studies, using clinical trial methodologies when possible, should be carried out to confirm these findings and determine the optimal probiotics to use.

Prebiotics

Prebiotics are non-digestible food components that benefit the host by selectively stimulating the growth and activity of microorganisms. Studies have shown that fibers and oligosaccharides can improve immunity and metabolism (8) and that the treatment of pregnant and lactating mice increases the proportions of *Lactobacillus* and *Clostridium leptum* and promotes a long-term protective effect against FA in the offspring (120).

Studies evaluating the effect of fiber/oligosaccharide intake in modulating asthma (121–123) have shown heterogeneous results, with one study reporting a reduction of wheezing (121) but others reporting no effect (122, 123). A recent Cochrane review has shown that although the addition of prebiotics to infant food may reduce the risk of eczema, it is not clear whether their use may affect other allergic diseases including asthma (124).

Synbiotics

When the use of a combination of prebiotics and probiotics produce synergistic health benefits it is described as a symbiotic. In FA mice models, both the microbiome and diet can affect the development of food tolerance by the induction of Treg cells (34). In cow's milk allergy, it has been demonstrated that treatment with extensively hydrolyzed casein formula plus *L. rhamnosus* GG promotes tolerance through changes in the infant gut microbiome (89).

A recent meta-analysis has shown their beneficial effects for eczema treatment (125). However, further well-conducted, randomized, placebo-controlled longitudinal studies are still needed in this area (126).

CONCLUSION

The microbiota is a highly dynamic environment influenced by multiple environmental and dietary factors, with a complex role in allergic diseases. Further studies with larger number of well-characterized patients and controls are needed to dissect the role of microbiome in allergic diseases are the performance. Despite some limitations, interventions with probiotics, prebiotics, and/or synbiotics show promise for the development of a preventive therapy, either by restoring altered microbiome functionality due to dysbiosis or as a boosting of immunological system in specific immunotherapy. However, the field is still relatively new and we expect many key findings to be made in the next few years. Detailed prospective, randomized, placebo-controlled studies will be essential for this purpose.

AUTHOR'S NOTE

All authors belongs to the Immunology Committee of the Spanish Society of Allergy and Clinic Immunology (SEAIC).

AUTHOR CONTRIBUTIONS

CM, MP and MP-G conceived and designed this manuscript and were involved in manuscript production contributing equally to

this work. TC, MME, MNLL, OL, LM, VM, ES, MZ and ML have read, revised and approved the manuscript.

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Immunological Tolerance and Function: Associations Between Intestinal Bacteria, Probiotics, Prebiotics, and Phages

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Post-birth there is a bacterial assault on all mucosal surfaces. The intestinal microbiome is an important participant in health and disease. The pattern of composition and concentration of the intestinal microbiome varies greatly. Therefore, achieving immunological tolerance in the first 3–4 years of life is critical for maintaining health throughout a lifetime. Probiotic bacteria are organisms that afford beneficial health effects to the host and in certain instances may protect against the development of disease. The potential benefits of modifying the composition of the intestinal microbial cohort for therapeutic benefit is evident in the use in high risks groups such as premature infants, children receiving antibiotics, rotavirus infections in non-vaccinated children and traveler's diarrhea in adults. Probiotics and prebiotics are postulated to have immunomodulating capabilities by influencing the intestinal microbial cohort and dampening the activity of pathobiont intestinal microbes, such as *Klebsiella pneumonia* and *Clostridia perfringens*. *Lactobacilli* and *Bifidobacteria* are examples of probiotics found in the large intestine and so far, the benefits afforded to probiotics have varied in efficacy. Most likely the efficacy of probiotic bacteria has a multifactorial dependency, namely on a number of factors that include agents used, the dose, the pattern of dosing, and the characteristics of the host and the underlying luminal microbial environment and the activity of bacteriophages. Bacteriophages, are small viruses that infect and lyse intestinal bacteria. As such it can be posited that these viruses display an effective local protective control mechanism for the intestinal barrier against commensal pathobionts that indirectly may assist the host in controlling bacterial concentrations in the gut. A co-operative activity may be envisaged between the intestinal epithelia, mucosal immunity and the activity of bacteriophages to eliminate pathobiots, highlighting the potential role of bacteriophages in assisting with maintaining intestinal homeostasis. Hence bacteriophage local control of inflammation and immune responses may be an additional immunological defense mechanism that exploits bacteriophage–mucin glycoprotein interactions that controls bacterial diversity and abundance in the mucin layers of the gut. Moreover, and importantly the efficacy of probiotics may be dependent on the symbiotic incorporation of prebiotics, and the abundance and diversity of the intestinal microbiome encountered. The virome may be an important factor that determines the efficacy of some probiotic formulations.

Keywords: immunological tolerance, probiotics, prebiotics, *Lactobacilli*, *Bifidobacteria*, inflammation, bacteriophages

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HISTORICAL PERSPECTIVE—MICROORGANISMS AND IMMUNITY

The mid seventeenth century ushered the beginning of a new era in medicine with the discovery of a microscopic world of bacteria and fungi (1) that in the twenty-first century these entities have been demonstrated to play a crucial role in shaping human immunity and maintaining immunological and metabolic tolerance throughout a lifetime (2, 3).

The history of health assertions relative to live microorganisms present in food is extensive and predates antiquity. Particularly, that involving lactic acid producing bacteria as described in the Old Testament from Persian scriptures where it was alluded to, that Abraham's consumption of sour milk provided a health and longevity benefit (4). Furthermore, writings from the Roman historian *Plinius* from the first-century, suggested that the administration of fermented milk foods could be used to treat gastroenteritis (4). Microbiological observations (5–7) and Metchnikoff (7) are credited with promoting the extensive health effects and restorative shifts in the balance of the gastrointestinal microbial cohort with the consumption of lactic acid bacteria. Metchnikoff who was at the Pasteur Institute, suggested that an extension of the life span of Bulgarian workers resulted from the ingestion of fermented milk foods (7). The consequence of consuming *Lactobacilli* inoculated yogurts could lead to a significant decrease of intestinal toxin-producing bacteria and that this effect then led to an increase in longevity. Moreover, Tissier (5, 8) reported that *Bifidobacteria* predominated in the intestines of breast-fed infants (5) and subsequently endorsed the administration of *Bifidobacteria* to infants distressed with diarrhea, and then further suggested that *Bifidobacteria* superseded the putrefactive gut pathobionts that would cause disease (8).

The early studies on lactic acid bacteria became recognized as the link between microbiology and the foundation of immunology (9). Metchnikoff's immunological studies credit his discovery of phagocytosis by macrophages and microphages as the elucidative step in host-defense mechanisms that established innate immunity; simultaneously Ehrlich defined the side-chain theory of antibody formation and the immunological pathways of how antibodies counteract toxins that encourage bacterial lysis. Furthermore, Erhlich also documented on how with the participation of complement, it led to an enhanced understanding of humoral adaptive immunity (9).

The early studies on lactic acid bacteria became recognized as the link between microbiology and the foundation of immunology (10). Metchnikoff's immunological studies associated with phagocytosis with macrophages and microphages resulted as an important discovery step in host-defense mechanisms. These studies established the foundation of cell innate immunity. Simultaneously Ehrlich explained the side-chain model of antibody formation and the immunological pathways of how antibodies counteract systemic toxins and encourage bacterial lysis. Furthermore, Ehrlich proposed that the participation of complement enhances the understanding of humoral adaptive immunity (10). Recent advances describe an overview of immunity as divided into two predominant systems that are determined by the speed and specificity of the reactions that occur (11). That is innate immunity describes chemical, physical, and microbiological barriers that usually encompass immune system elements of neutrophils, monocytes, macrophages, and the complement network of cytokine proteins and other acute phase proteins that provide an immediate response to an infective insult, a response that is imperative for survival. Cell mediated immunity is the immune characteristic exhibited by higher animals that comprises antigen specific responses through T and B lymphocytes, a slow but precise response to an infective agent (12). Moreover, a link has been recognized to exist between the innate and cell mediated immunity systems that is necessitated to complete the immune response and that is the role of complement that effectively bridges both systems in order to neutralize an infective bacterial, viral or other insult (13). The human microbiome project (14) has redefined the role of bacteria that live on and within humans especially in the intestinal tract, from one that was considered to be a site of toxic waste and pathogenic bacteria to a site with important immunological activity (15). Recently, reviews have described how the role of probiotics enhancing immunological functions in the intestines to restore local and extra-intestinal mucosal and innate immunity equilibrium have gained significant support (16, 17).

DYSBIOSIS

A historical perspective on the origin of the term "dysbiosis" has been recently documented (18), with Haene (19) a German microecologist, credited with popularizing the term (18). Moreover, Haenel was also instrumental in contrasting dysbiosis with eubiosis which he referred to this latter term as the "normal state" (19).

Dysbiosis usually refers to an imbalance of intestinal bacteria that occupy the lumen of the gut that refers to adverse microbiome pattern shifts that have been associated with disease development and progression (18). Further, dysbiosis has been reported to be the subject of multiple explicit and semi-explicit delineations (18).

The chronic diseases that have been associated with intestinal microbiome dysbiosis include intestinal inflammatory diseases (e.g., Ulcerative colitis, Irritable Bowel Syndrome), auto-immune diseases (e.g., multiple sclerosis, asthma), metabolic diseases (e.g., diabetes), neuro-degenerative diseases (e.g., Parkinson's Disease, Dementias), and cancer (20). The community of bacteria that inhabit the intestines has been investigated from multi-dimensional studies with samples from different geographical areas. The samples were constructed from variations observed in the concentration of three dominant/conserved bacterial genera namely, *Bacteroides* (enterotype 1); *Prevotella* (enterotype 2); and *Ruminococcus* (enterotype 3) (21, 22). The topographical view of the intestinal microbiome has provided the basis to further understand the bacterial phyla configurations associated with dysbiotic shifts that have been correlated with disease states. As

for example, when comparing the intestinal microbiome of obese to lean subjects studies have shown increased phyla abundances in *Firmicutes* and decreased *Bacteriodetes* (23).

INTESTINAL BACTERIA AND IMMUNITY

The human microbiome project has largely changed the way bacteria have been viewed in the large bowel; from a collection of waste products and pathogens to a more pragmatic view concerned with early immunological and metabolic development and to sustain a stable equilibrium. The mucosal surfaces of the intestines provides a large complex and interactive surface area between the commensal bacterial cohort and the intestinal epithelia (24). Specifically, the complex nature of the intestinal architecture is evident from the components of the differentiated epithelial cell types that are encountered such as enterocytes (approximate turnover of 3–5 days which migrate out of the aberrant crypt), enteroendocrine cells, goblet cells, tuft cells, and Paneth cells (approximate turnover of 30 days which do not migrate out of the aberrant crypt) (25, 26). The cross-talk that has evolved between bacteria and host gut tissue has spanned millennia (16). In particular, Paneth cells synthesize and secrete proteins and antimicrobial peptides (i.e., α/β defensins; cathelicidin; 14β-glycosidases; C-type lectins; ribonuclease), activities that emanate from various external and internal stimuli (e.g., intestinal bacterial milieu such as bacterial surface components) and toll-like receptor activity (27).

Experiments with gnotobiotic murine models have shown that when colonizing the animals with a single bacterium that adhered to the surface of the intestinal epithelia encouraged the growth and activity of a set of genes in the host animal, that were involved in immune function, and the elaboration of proteins that protected against the deterioration of the gut epithelia [**Figure 1**; (28, 29)] Additional experiments have also reported on the interactions between commensal bacteria and host immune cells and have shown that macrophages that are in close proximity to the base of the intestinal epithelia participate in antigen recognition and responses (30). That is, macrophages/dendritic cells take up antigen and produce cytokines and depending on which cytokine is produced, a response is elicited such as anti-inflammatory. Research has advanced the idea that antigens from the intestines or from the environment are transcytosed (31, 32) by specialized enterocytes namely, Microfold-cells into the sub-epithelial dome region of the Peyer's patch mucosa, an area rich in macrophages/dendritic cells (33–35) where these antigen-presenting cells reside and progress to collect the trancytosed bacteria and macromolecular antigens (36). In the *lamina propria* macrophages have been demonstrated to sample antigens, entero-pathobionts and commensal bacteria through trans-epithelial dendrites. Intestine-resident macrophages (CX3 CR1hi^{+}) that are resultant from blood monocytes (Ly6C^{+}) do persist in close physical proximity whilst maintaining the integrity of the intestinal epithelial cells and barrier function. Experiments investigating specific effects with the human and animal commensal bacillus, *Clostridium butyricum,* have demonstrated that in a murine model the spore forming anaerobe induced IL-10 producing macrophages that suppressed an acute experimental inflammatory outcome (37). The explicit effect triggered by *Clostrodium butyricum* treatment showed that the outcome was not associated with cytokine IL-10 production by Treg cells but that instead the anti-inflammatory effect was due to IL-10-producing F4/80+CD11b+CD11c^{int} macrophages through the TLR2/MyD88 pathway and subsequently observed to have accumulated in the inflamed mucosa. The posit that macrophage sampling eliciting and then escalating an antigen specific immune response has recently been insightfully progressed (38, 39). Man et al. (38) in a murine study with *Salmonella typhimurium* demonstrated that there also exist protective mechanisms in the intestines that hinder the access of bacteria to the intestinal epithelia that thereby block pathogen penetration across the intestinal barrier. Specifically, intestinal epithelium cell emerging signals, trigger the intraluminal migration of CX3CR1^{+} cells forming an intricate network of cellular defense. This protective effect presents an additional barrier to the copious amounts of mucus and Immunoglobulin A that are constantly produced to maintain local homeostasis, when these first line of defense are breached the complex network of intraluminal migration prevents early local infectivity from progressing.

It is widely acknowledged and published in the scientific literature that food antigens do indeed affect immunological responses (40). In the intestinal induction of Tregs and Th17 is characteristic of the gut immune network (41). Consequently, the Th17 cells strengthen the mucosal barrier and concomitantly encourage intestinal epithelial cells to produce antimicrobial peptides, with the overall effect centered on maintaining local homeostasis (42). Regulatory T cells that promote tolerance do so to reduce reactivity to dietary and environmental antigens by decreasing intestinal inflammation. Strikingly this regulated pro-inflammatory to anti-inflammatory activity has been demonstrated to be a cooperative crosstalk between commensal bacteria, the host intestinal epithelia and the mucosal immune network to reduce the risk of inflammatory responses by down regulating the effect of dietary and or bacterial antigens that in turn maintains local immunity in equilibrium (43). For example the mucosal accumulation of Th17 cells has been shown to be subject to stimulation by commensal bacteria (i.e., Segmented Filamentous Bacterium) (44) indicating that commensal bacteria very much promote the accumulation of Th17 cells in the intestinal *lamina propria* (45, 46).

As a consequence, specific bacterial species have been shown to significantly influence immune tissue subsets of cells and maturation of innate immunity in health and disease [**Table 1**; (70, 71)]. Probiotic bacteria, in one instance, were posited to induce a re-regulation of the nuclear translocation of NFκB in intestinal epithelial cells by shaping the release of TNF-α that links this molecular action to a significant decrease in epithelial permeability and susceptibility to an inflammatory Crohn-like ileitis in the SAMP1/YitFc murine model that spontaneously progress to an inflammatory disease (72).

Among all the microbial metabolites expressed in the intestinal lumen the most abundant in the colon are SCFAs (73). Recently, probiotic species from the *Lactobacilli* and

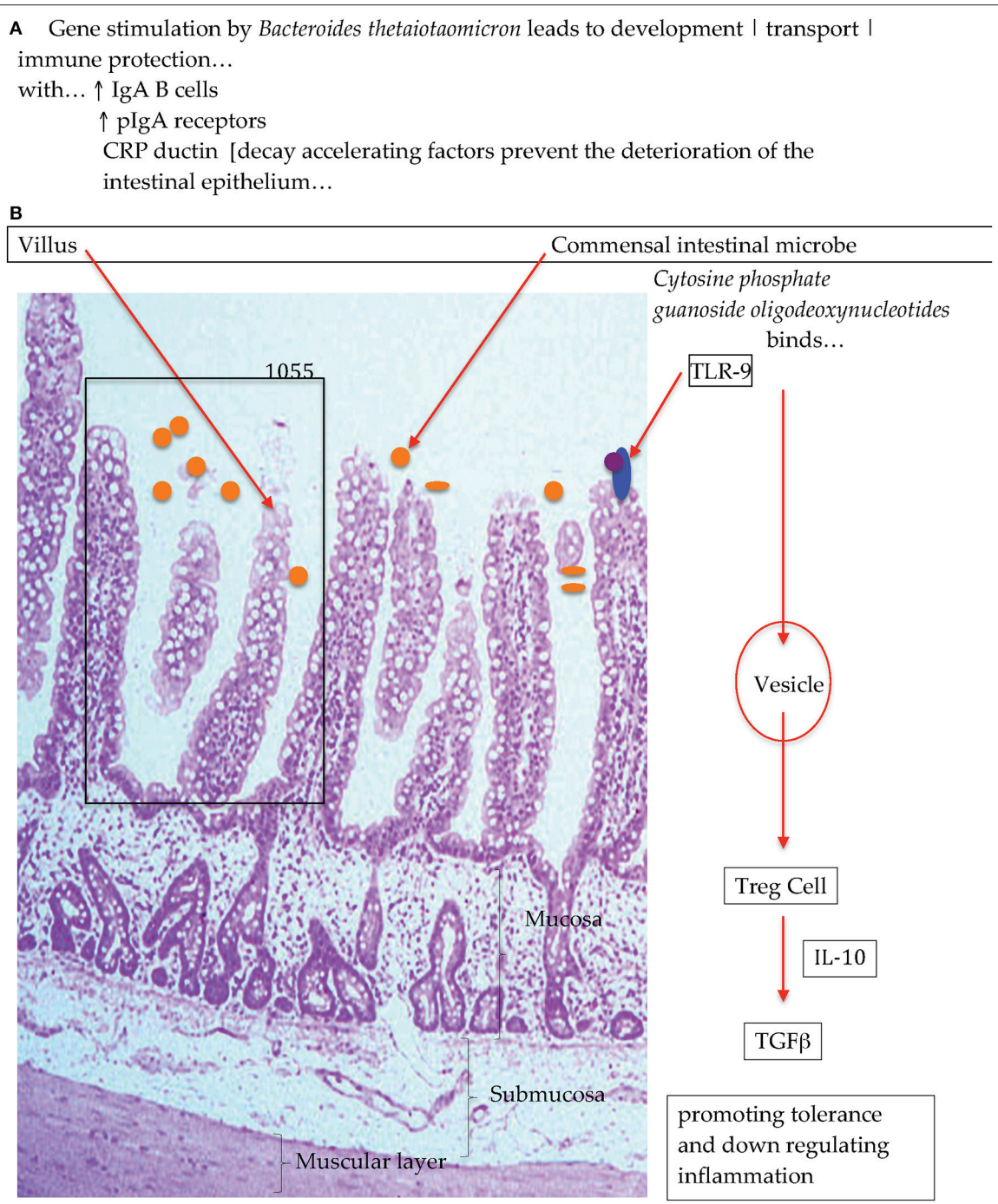

FIGURE 1 | Diagrammatic representation of **(A)** bacterial colonization stimulates intestinal gene activation and **(B)** commensal bacteria interactions with Toll-Like Receptors to promote immunological equilibrirum (28).

Bifidobacteria genera were demonstrated to increase the levels of fecal SCFAs (74). These chemical species exhibit multi-faceted regulatory roles in the local mucosal immune system in the gut. That is (i) SCFAs are a major energy source of intestinal epithelia significantly influencing gene expression (i.e., epigenetics) that is an important pre-requisite for maintaining the coherence of the epithelial lining and epithelium to epithelium tight junctions and mechanisms of defense. Hence it is envisaged that SCFAs can also (ii) regulate local mucosal derived innate immune cells such as macrophages or dendritic cells as well as neutrophils. Moreover, SCFAs also (iii) are involved in a bi-directional regulation of specific antigen-triggered adaptive

TABLE 1 | Commensal intestinal bacteria and effects on the development of tolerogenic macrophages|dendritic cells; induction of Treg cells; and stimulation of TLRs.

Intestinal bacteria	Immune tissue induction effects	References
Segmented Filamentous Bacteria Gram positive, spore-forming obligate anaerobe	In the terminal ileum - Alter T cell subsets - Induce accumulation of Th17; induce serum amyloid A—a protein that acts on the *lamina propria* and stimulates Th17 inducing environment - Can drive an autoimmune disease	(47)
Bacteroides fragilis Gram negative, obligate anaerobe	In the large bowel [colon] (48) - Direct development of FoxP3+ Tregs - Bacterial *polysaccharide A* mediates conversion of CD4+T cells → FoxP3+Tregs - TLR2 signaling in T cells mediates this effect and not in macrophages|dendritic cells - *Polysaccharide* A–TLR2 $^{-/-}$ co-operations results in ↑ formation and release of IL−10 by Tregs and notably ↓the multiplication of Th17 cells in the intestines - PSA derived from this commensal is a symbiosis factor that promotes immunologic maturation within mammalian hosts	(49)
Clostrodium cluster IV and Cluster XIVa	Promote expansion of colonic and systemic Treg cells - Clostridia activate intestinal epithelial cells secretion of TGFβ and Treg inducing molecules MMP2/MMP9/MMP13/indoleamine 2,3-dioxygenase ↑ Tregs in the colon only - Clostridia in the intestines suggest an effect to multiple sites systemically - Tregs ↑ spleen/liver/lungs - Clostridia induction of Tregs PRRs - Mice deficient in MyD88/Rip2/Card9 have normal Tregs numbers in the colon - ↓ levels of systemic IgE/IL−4 and ↑ levels of ↑ levels of IL−10 producing splenocytes are found in experimental animal model of OVA–induced asthma in the presence of Clostridia	(50)
Bacteroides thetaiotaomicron	In the colon - Binding of immunoglobulin A, a requisite for *B. fragilis* (and other commensal intestinal bacteria) to occupy a defined mucosal niche in the intestines - Maintain host-microbe mutualism	(29) (51)
Lactic acid bacteria overall	Weak induction IL-12|TNF-α weak	(52)
Lactic acid bacteria [most species]	- Strong induction IL-12|TNF-α weak	(53) (54)
	- ↑Treg cells producing ↑levels IL-10 (variable levels)	(55)
Lactobacilli|Bifidobacteria	- Increased FoxP3+ Tregs|suppressor activity - Modulate dendritic cell function	(56) (57)
L. rhamnosus GG	- ↑FoxP3+ Tregs|TGF-β Tregs|TGF-β production	(58, 59)
L. casei	- ↑FoxP3+ Tregs|IL-10|TGF-β production	(60)
L. reuteri	- Prime monocyte-derived dendritic cell|DC maturation dendritic	(61)
L. paracasei	cell|DC maturation |↑IL-10|↑FoxP3+ Tregs|activation of TLR-9	(62)
L. plantarum	- ↑FoxP3+ Tregs|IL-10|TGF-β production	(63)
L. acidophilus	- ↑FoxP3+ Tregs|IL-10|TGF-β production	(64)
	- ↑IL-10|↓IL-4|Treg-associated TGF-β production	(65) (66)
	- ↑IL-10|↓IL-4|Treg-associated TGF-β production	
B. lactis	- Prime neonatal macrophages|dendritic cell	(67)
B. bifidum	- ↑FoxP3+ Tregs|↓TNF-α|IL-6	(68)
B. infantis	- ↑IL-10 production macrophages|DC	(69)
B. longum *B. breve*	- Activation TLR-2|dendritic cell maturation/activation|↑IL-10 production	(10)
E. coli Nissle 1917	- ↑FoxP3+ Tregs	

immunity activities that are mediated by T and B lymphocytic cells.

PROBIOTICS

Probiotics were originally defined as live micro-organisms that could be added to fermented foods that in such a matrix could be advantageous to health by establishing an overall improved stability to the intestinal microbial cohort (75) and then modified by the FAO/WHO (76). Micro-organisms predominantly utilized as probiotics include various members of the *Lactobacilli* or *Bifidobacteria* species which are administered either individually or combinations of various formulations. The non-pathogenic yeast, *Saccharomyces boulardii*, has also been designated as a probiotic following its administration in both animal studies and human clinical trials. Further, the inability of probiotics to permanently colonize the intestines has let to posits that they be dispensed in sufficient quantities to maintain high amounts in the colon; and that probiotic species be of human origin.

Probiotics: *in vitro* and Laboratory Animal Data

The critical and important purported health-recommended effect of probiotics is the ability of these bacteria to enhance mucosal immune defenses (77). The gut associated lymphoid tissue can be distributed according to anatomical sites, with lymphocytes disseminated throughout (i) intestinal epithelia regions in contact with the *lamina propria* and (ii) structured lymphoid tissue sites, that includes Peyer's patches and mesenteric lymph nodes (35).

Studies with germ-free animals (gnotobiotic) clearly demonstrate that in the absence of significant intestinal microbial colonization, the effective component of the mucosal immune system remains immature, an outcome that enhances the host's susceptibility to bacterial infections by pathobionts (78). Moreover, general mechanisms for the function of probiotics have been associated with protective effects provided against pathobiont microbial colonization and translocation within the intestines (79). It has been postulated that mechanisms include production of antibiotic type substances (i.e., reuterin) (80) and competition for receptor sites on the mucosal intestinal surface (81). Other mechanisms such as heightening immune defenses of the host that produce adjuvant effects, amplified immunoglobulin A production and cytokine stimuli, as well as competition with pathogenic organisms for intraluminal nutrients (82, 83). Studies have also suggested that probiotics that include non-immune intestinal host defenses could strengthen tight junctions of the gut mucosa, increase mucous secretions, enhance motility, and produce amino acid by-products including arginine and glutamine, and SCFAs, that could secondarily function as protective foods for the gut (84–87).

A series of basic laboratory studies have highlighted the influence that probiotic bacteria and commensal bacteria may have on the maturation of intestinal macrophages/dendritic cells and the production of various cytokines (**Table 2**).

In vitro and *in vivo* studies have appraised the effects of probiotics on the prevention/development of large bowel cancer (i.e., specifically in the colon) (96). Epidemiological studies have associated large bowel cancers with genetic, environmental risk factors, including diet and the nature of the intestinal bacterial cohort (97). Probiotics used in animal models have been shown to reduce the occurrence of precancerous lesions observed in aberrant crypts (98, 99). *In vitro* experiments with probiotics have further suggested that the administration of these beneficial bacteria could reduce hypertension and lower serum cholesterol (100, 101). Furthermore, animal studies have also suggested that *Helicobactor pylori* infection in germ free murine models could be averted with the administration of *Lactobacilli* to dislodge *H. pylori* from the stomach and that cell attachment and invasion by enteropathogenic bacteria such as *Escherichia coli* and other gram-negative pathobionts can be inhibited with the use of *Lactobacillus acidophilus* containing probiotics (102). Interestingly, in a murine model of experimental uremia (103) intestinal macrophages were skewed toward a pro-inflammatory phenotype with reduced phagocytic activity with resultant bacterial translocation that triggered a local inflammatory response. The administration of a *Lactobacilli* LB probiotic reduced bacterial translocation by improving macrophage phagocytic activity.

Probiotics Human Studies

The extensive body of clinical evidence that supports the use of probiotics in the prevention or treatment of gastrointestinal diseases have been administered in clinical trials with pediatric and adult patients (104–107). Formulations with probiotic bacteria include members from the bacterial genera, *Lactobacilli, Bifidobacteria,* and *Streptoccocus* (i.e., *Streptococcus thermophiles*) or the yeast, *S. boulardii* (108). Numerous studies report that probiotics provide efficacy for reducing the risk of developing Clostridium-Difficile-Associated-Diarrhea (109); preventing antibiotic associated diarrhea (110, 111); and Traveler's diarrhea (112, 113). Moreover in a systematic review study of outpatients, *S. boulardii* was reported efficacious in preventing antibiotic associated diarrhea (110). Hence probiotics can have beneficial effects on diarrheal conditions and related gastrointestinal symptoms. Further, evidence-based probiotic formulations can be administered to either prevent or reduce the severity of pathogenic bacteria triggered intestinal inflammations. Consequently, inflammatory bowel diseases (IBDs) can present with major clinical inflammatory associated complications. Anti-inflammatory pharmaceutical agents have been used extensively to ameliorate the chronic inflammatory responses that occur associated with IBDs. *In vivo* laboratory studies have reported success with probiotics used to prevent or reduce the inflammatory response associated with colitis (114). Although encouraging, additional studies are needed and warrants further focused research to make conclusive inferences on the efficacy of probiotics for ulcerative colitis, Crohn's Disease, and liver diseases (e.g., NAFLD) (115).

Mechanistically, it has been posited that formulas supplemented with probiotic bacteria could induce changes in the stool pattern that is bifidogenic and that it could mimic

TABLE 2 | Probiotic and prebiotic induction of regulatory T cells.

Prebiotics	Probiotic|Commensal microbial effects	References
FOS	↑*Bifidobacteria* genus	(88)
	↑levels IL-10|macrophages|dendritic cells expressing TLR2 and TLR4	(89)
GOS	↑*Bifidobacteria* genus	(90)
	↑Tregs|↑levels IL-10|↓IL-6|↓IL-1β|↓TNF-α	(91)
Inulin	↑*Bifidobacteria* genus|↑*Lactobacilli* genus|*Faecalibacterium prausnitzii*|*Eubacterium* spp.	(92)
	↑levels IL-10|dendritic cells	(93)
Resistant starch	↑Firmicutes phyum	(94)
	↑Tregs|spleen|Peyer's Patches	(95)

that observed with breast-fed infants (116, 117). Alternatively, in an early study with undernourished Peruvian infants, especially among non-breast fed children with a high encumbrance of diarrheal disease, the administration of *Lactobacilli* GG was reported associated with significantly fewer episodes of diarrhea (118). Clinical efficacy with probiotic formulations have been reported to reduce the number of episodes of diarrhea and rotavirus shedding among chronically infected infants admitted to hospital, young children as well as adults (119). Studies specifically with *Lactobacillus* GG administered as a treatment modality, during acute rotavirus infections with diarrhea has been reported and associated with higher titres of polymeric immunoglobulin A to the infections with rotavirus (119, 120). Additionally, significant experimental and clinical studies have reported the efficacy for reducing the incidence of neonatal necrotizing enterocolitis with the administration of *Lactobalcilli* and *Bifidobacteria* (121, 122). Increased efficacy of probiotics have recently been reported to significantly decrease the risk of developing clinical complications related with necrotizing enterocolitis and sepsis; decrease mortality and length of hospital stay; as well as promote neonatal weight increases in very low birth weight infants (123). Further that probiotics were more efficatious when administered with breast milk and or an infant formula, and consumed for <6 weeks, provided in a dose of $\sim 10^9$ CFU/day and the probiotic formulation included multiple strains (123).

PREBIOTICS

Prebiotics, have been defined as non-digestible food components (i.e., non-digestible carbohydrates), are important functional foods that potentiate the action of commensal/beneficial bacteria in the intestines [**Table 2**; (124)]. Hence the effectiveness of prebiotics is largely dependent on these substances eluding hydrolysis and absorption in the proximal small intestines so as to reach the large bowel (125). Once in the large bowel to be utilized selectively by the commensal group of bacteria (124). These include, Fructooligosaccharide (FOS), Galactooligosaccharide (GOS), inulin, dietary carbohydrates, and Xylooligosaccharide (XOS) are among the most commonly studied prebiotics in clinical studies (126, 127). Interestingly, oligosaccharides in human breast milk have been reported to represent the quintessential prebiotic, as they can readily facilitate the favored growth of the *Bifidobacteria* and *Lactobacilli* genera in the large bowel of neonates that have been exclusively breast-fed (128).

The chemical structures of prebiotics prevents metabolism and absorption in the small bowel and leads to bacterial fermentation reactions in the large bowel (i.e., specifically the colon) to form combustible gases, lactate, and SCFAs (i.e., acetate, propionate, butyrate) (129) that have been associated with health benefits (130).

Prebiotics: *in vitro* and Animal Experimental Data

Strong clinical evidence pertaining to the potential health benefits of prebiotics, result from *in vitro* and *in vivo* study models (131–133) *in vitro* studies have shown that individual specific bacterial species from the *Bifidobacteria* and *Lactobacilli* genera will ferment selected prebiotics as defined by the production of SCFAs in an acid environment (134). The mechanism of this selective activity involves factors that include the lowering of colonic pH and the production of metabolites that can inhibit pathobiont growth while simultaneously promoting the growth of probiotic bacteria and the production of antimicrobial effects (135, 136). Studies have shown the preferential administration of prebiotics over probiotics for the selected growth of bacteria in the large bowel (137, 138). For example, the incorporation of oligosaccharides in doses of 5–7 g/day may lead to the proliferation of certain types of bacteria that are generally considered to be beneficial (i.e., *Bifidobacteria*, *Lactobacilli*, non-pathogenic *E. coli* while decreasing *Bacteroidaceae*) to the detriment of pathobionts; this re-equilibration or intestinal homeostasis of the colonic biotope has been designated as the *prebiotic effect* (139).

The effect of prebiotics on the proliferation of specific bacterial classes is complex. The interactions with the intestinal microbiome cannot be easily explained by prebiotic compounds acting as exclusive substrates (140). Exploiting intestinal bacterial communities through the introduction of prebiotics has the capacity to indirectly influence beneficial immune responses (141). The production of SCFAs the major products of bacterial fermentation of prebiotics in the absence of oxygen modulates the concentration of SCFAs by down regulating pro-inflammatory mediators by intestinal macrophages (142).

SCFAs such as propionic acid and butyric acid have been reported to inhibit molecular induced expression of adhesion

molecules, chemokine formation with concomitant suppression of monocyte/macrophage immune activities and neutrophil recruitment that in combination suggest an anti-inflammatory effect (142). This effect is articulated by butyrate, which is reported to suppress lipopolysaccharide and cytokine-promoted production of pro-inflammatory mediators including TNF-α, IL-6 and nitric oxide while enhancing the release of IL-10 an anti-inflammatory cytokine (143–145). Interestingly SCFAs have been documented to decrease the *in vitro* adherence of monocytes and lymphocytes to human umbilical vein endothelial cells (146). Furthermore, butyric acid interactions with monocytes, has also been shown to reduce the constitutive and IFN-γ-induced expression of lymphocyte function-associated antigen 3 and intercellular adhesion molecule-1 (147). In addition SCFAs regulate several functions expressed by leukocytes and these include production of a number of cytokines (i.e., TNF-α, IL-2, IL-6, and IL-10), eicosanoids and chemokines (e.g., macrophage chemo-attractant protein-1 and cytokine induce neutrophil chemo-attractant-2) (148).

SCFAs have also been reported to modulate the production of prostaglandin E2 (PGE2) and that this activity stimulates the *in vitro* production of PGE2 by human monocytes (149). This eicosanoid has been shown to suppress T cell receptor signaling and may play an important role in the resolution of inflammatory responses toward equilibrium (150), redefining it as an anti-inflammatory prostanoid by attenuating the formation of IL-1β and TNF-α by macrophages and Th1 differentiation (151). SCFAs such as acetic acid and propionic acid have been reported to reduce TNF-α formation induced by LPS that have been stimulated by human neutrophils (152). Moreover, reports relative the effects of propionate and butyrate demonstrate an inhibitory expression profile of pro-inflammatory mediators (i.e., TNF-α, CINC-2αβ, NO) from rat neutrophils through the attenuation of NF-kB activation (153). Prebiotics such as FOS, GOS, inulin and resistant starch have been demonstrated to affect microbial genera (indigenous *Bifidobacteria*, *Lactobacilli, Faecalibacteria*) (154, 155) that progress the up-regulation of Tregs by different mechanistic pathways and in turn re-regulate pro-inflammatory activity (94).

Prebiotics influencing immune modulation by exploiting the metabolic activities of commensal intestinal bacteria where experimental murine models suggest that such activity can significantly reduce the precancerous colonic lesions present as aberrant crypt foci (156). Other studies have documented that prebiotics augment the bioavailability and absorption of minerals such as calcium and may affect the metabolism of other minerals namely, magnesium, iron, and zinc (157); stimulating the reduction of endogenous carcinogens such as sialomucin (158); and reducing the growth of tumors in murine models of carcinogenesis (159).

PREBIOTICS AND HUMAN STUDIES

Clinical studies have confirmed that FOS has a bifidogenic effect on the human large bowel (i.e., colon) and the endogenous intestinal microbiome (160, 161). Moreover, a number of clinical studies have also demonstrated the bifidogenic effect for inulin-type fructans (162). These studies have effectively demonstrated the growth promoting activity of prebiotics and the targeting of the bacterial genera *Lactobacilli* and *Bifidobacteria* (163). The overall implications from the clinical data is that the proposed bifidogenic effect is not simply attributed to prebiotics as preferential substrates for the commensal bacterial cohort, rather that prebiotic substances can interact with other commensal bacteria and may be subject to associations with environmental fluctuations including variations in luminal pH, and other unknown factors in order to achieve a net bifidogenic effect in the large bowel (164). In a clinical study with children attending day care daily supplementation with oligofructose (dose: 2 g/day over 3 weeks) was associated with significantly fewer episodes of diarrhea, flatulence, vomiting and fever, and with reductions in the level of pathobionts from the *Clostridia* and *Staphylococci* genera and with increases in *Bifidobacteria* genus (165). Infants supplemented with formula that included a prebiotic mixture (dose: 8 g/L) achieved normal growth and stool features that were more similar to those of breast-fed infants and in comparison with infants fed an un-supplemented formula (166). Others have reported no effect for oligofructose-supplemented infant cereal (167). Studies on the effect prebiotics have on the intestinal microbiome have reported that the consumption, daily, of whole-grain-wheat was observed to provide a pronounced prebiotic effect on the composition of the intestinal microbiota, positing that this activity may contribute toward beneficial physiological effects (168). Other studies have reported that prebiotics in the form of a whole-grain-maize augmented cereal (dose: 48/day) induced a bifidogenic modulatory effect on the intestinal microbiota (169). No significant changes were observed in serum lipid profiles, blood glucose levels or fecal output measures (169). Celiac disease is an autoimmune inflammatory problem characterized by the interplay between the host's genetic factors and gluten as the environmental trigger (170).

Clinical studies have posited and reported that the concentration of SCFAs in blood and more importantly in the intestines may predispose to or prevent pathological conditions such as IBD (171), cancer and obesity (172), diabetes (173), and symptoms such as diarrhea (174). Interestingly, in a study with healthy physically active subjects a synbiotic supplement, increased fecal *Lactobacillus paracasei* with no appreciable effect on mucosal immunity (175). This latter outcome is not surprising given that in healthy individuals mucosal immunity should be in equilibrium.

Clinical studies administering prebiotics to encourage intestinal microbiome shifts toward the production of increased levels of SCFAs remains contentious though with clinical studies, showing a benefit with beneficial microbial shifts and immune function (94, 176, 177) and others not (178).

BACTERIOPHAGES

Bacteriophages are ancient dependent infective agents that have existed on this planet for millennia, were discovered about a century ago (179), and have been posited to participate in

immunological activities of the host (180). The predominant intestinal phage load harbored by adult healthy individuals has been reported to be a member of the order *Caudovirales,* a double stranded DNA bacteriophage (from the families *Podoviridae*, *Siphoviridae,* and *Myoviridae*), single stranded DNA bacteriophages (from the families *Microviridae* and *Inoviridae*) and RNA viruses (181–184). Viruses that reside in the intestines are comprised predominantly of bacteriophages (including prophages) and to a lesser degree eukaryotic viruses, reported to be stably integrated into bacterial genomes (parasitized phase) and lytic phages which can infect and lyse bacteria and hence release virus particles (185). Bacteriophages can hence influence the bacterial structure of the microbiome through a parasitic or lytic phase of bacterial cells and show the greatest abundance and diversity in the intestines of healthy adults (186).

Recent reports show that the neonate is exposed to a diverse range of bacteriophages at birth (187), an exposure that together with bacteria could herald a further consideration of how immunological and metabolic tolerance is achieved in early life. Bacteriophage directed control of behaviors of how bacteria colonize and survive in different anatomical sites encourage developmental properties that helps establish commensal populations that reduces the risk of disease (182). Studies report that bacteriophages may be involved in important functions in human immunity by defending the intestinal epithelial barrier and mucosal tissue from infections by pathobionts (188).

Bacteriophages influencing the stability of the intestinal microbiome cohort indirectly shape the immunological and metabolic functions of intestinal immunity, especially as reported for the direct action on T and B cells (189). Bacteriophages have been reported to adhere to the mucus layer of the intestines, and that the interaction with glycan residues from mucin glycoproteins affords the viruses a niche that is in close proximity to the intestinal epithelial layer and mucosal surfaces (190). Furthermore, it is reported that when phages exhibit muco-adherent properties, such actions can influence the activity of the innate and cell mediated immune systems (191). Moreover, temperate phages that positively influence host immunity could do so by selective screening of the commensal bacterial cohort (191).

In concert with peptides that exhibit antimicrobial properties in the mucus layer there is demonstrated a control over the density of the commensal bacteria that can occupy the mucus layer. This effective local control provides a protective barrier against commensal pathobionts while establishing a symbiotic relationship with the host; an immunological defense mechanism not derived from the host.

In the pre-antibiotic era, the use of bacteriophages to treat infections was an early example of the immunogenicity effect that was attributed to bacteriophages (192). A number of studies have documented the immune-modulatory effects of bacteriophages. These have included, (i) an anti-bacteriophage immune response, whereby exposure to bacteriophages in the circulation have induced strong anti-bacteriophage humoral responses resulting in swift and proficient neutralization and clearance of the phage on subsequent exposures to the virus (193, 194); (ii) bacteriophage-mediated re-regulation of the over-production of reactive oxygen species by phagocytes is a critical immunological effect that significantly contributed to the favorable effects of bacteriophage therapy in patients diagnosed with a life-threatening condition such as sepsis (193); and iii) a chemically modified phage with high affinity ligands for cell specific receptors has been reported to induce humoral and cellular immune responses regressing solid tumors in murine models (193, 194).

Certain Eastern European countries with bacteriophage treatment centers, routinely administer phage therapies for the prophylactic and therapeutic treatment of bacterial infections for ulcers/wounds, septicemia, UTIs, MRSA and others (195). Reports note though that the state of the immune system can determine what type of an effect the virome may have that then determines the interactions that ensue with host immunity. This is particularly relevant with inflammatory diseases such Crohn's Disease and Ulcerative colitis where the intestinal virome has been deemed to be abnormally altered in terms of increased bacteriophage richness and then correlated to decreased bacterial diversity (196). Moreover the bacteriophage changes were Inflammatory Bowel Disease (IBD) specific. Such specific effects have been consistent with studies that have reported decreases in diversity and abundance of the bacterial phyla (197). Specifically decreased *Bacteroidetes* and *Firmicutes* phyla from the fecal samples, were reported associated with IBD (197).

It is hence very probable that bacteriophages may control and cull the bacterial population in the intestines with substantial turnover that as such significantly influences bacterial diversity/abundance and metabolism in the intestines. Furthermore, increases in the intestinal virome due to decreases in commensal bacterial diversity and abundance could also explain the inefficacy that has been reported with certain probiotic treatments for IBD, especially in patients diagnosed with Crohn's Disease.

SUMMARY

The commensal microbial cohort functions to develop and establish the host's immune system (i.e., mucosal and cell mediated) in order to promote immunological and metabolic tolerance. The microbiome complement effect is mediated by bacteriological factors that stimulate cells of the host; and these factors operate across a diverse set of host receptors and cellular molecular targets that are expressed on the surface or within the cells of the host. The host receptors that interact with microbial factors include numerous operators such as pattern recognition receptors (i.e., TLRs), receptors such as C-type lectin and nucleotide oligomerization and RIG-1-like receptors, which can sense important microbial macromolecular constituents such as nucleic acids (i.e., DNA, RNA), proteins and cell wall components (198).

Specifically, probiotics and prebiotics are reported to have positive immuno-equilibrium restorative effects. An increasingly supported posit is that bacteria such as those from the probiotic

genera of *Bifidobacteria* and *Lactobacilli* can participate in immune-regulation and do so by inducing regulatory T cells (199, 200). The beneficial immune-modulatory effects are elicited across several molecules, that include microbial cell walls, peptidoglycan, and exopolysaccharides, through interactions with specific host cell receptors (i.e., Toll-Like Receptor (TLR)-2 and TLR-4) (200). Relative to prebiotics, these compounds encourage the intestinal microbiome production of SCFAs, which have a central role in intestinal immunogenicity (201). It is generally accepted that SCFAs such as acetate propionate and butyrate can interact with local intestinal epithelial and mucosal immune tissues as well as having significant epigenetic effects (202), and are essential participants in health and disease. Bacteriophages seem to have a two-way role in the intestines that balances health and disease; from determinants of decreased abundance and diversity of the commensal microbiome to the control of bacterial diversity and abundance as a necessary factor to control pathobiont insults that balances the risk of disease and health. Bacteriophages, it would seem display immune-suppressive characteristics in the intestines across involvement in a number of immune related areas such as the control of inflammation and autoimmune reactions (189).

AUTHOR CONTRIBUTIONS

LV conception and design of the manuscript. LV, GV, and SH read, amended, and approved the final version of the manuscript.

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Delivery of IL-35 by *Lactococcus lactis* Ameliorates Collagen-Induced Arthritis in Mice

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IL-35, a relatively newly discovered cytokine belonging to the larger IL-12 family, shows unique anti-inflammatory properties, believed to be associated with dedicated receptors and signaling pathways. IL-35 plays a pivotal role in the development and the function of both regulatory B (Bregs) and T cells (Tregs). In order to further its therapeutic potential, a dairy *Lactococcus lactis* strain was engineered to express murine IL-35 (LL-IL35), and this recombinant strain was applied to suppress collagen-induced arthritis (CIA). Oral administration of LL-IL35 effectively reduced the incidence and disease severity of CIA. When administered therapeutically, LL-IL35 abruptly halted CIA progression with no increase in disease severity by reducing neutrophil influx into the joints. LL-IL35 treatment reduced IFN-γ and IL-17 3.7- and 8.5-fold, respectively, and increased IL-10 production compared to diseased mice. Foxp3$^+$ and Foxp3$^-$ CD39$^+$ CD4$^+$ T cells were previously shown to be the Tregs responsible for conferring protection against CIA. Inquiry into their induction revealed that both CCR6$^+$ and CCR6$^-$ Foxp3^{+or-} CD39$^+$ CD4$^+$ T cells act as the source of the IL-10 induced by LL-IL35. Thus, this study demonstrates the feasibility and benefits of engineered probiotics for treating autoimmune diseases.

Keywords: ***Lactococcus*, probiotic, IL-35, therapeutic, IL-10, cytokines, regulatory T cells, CCR6**

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, inflammatory, systemic autoimmune disease that affects about 0.24% of the worldwide population, and roughly 1% of the general population in Western countries. RA is two to three times more common in women than in men (1–5). RA manifests as a chronic synovitis and progressive destruction of the joints, leukocyte infiltrates, and cartilage destruction and bone erosion. Approximately half of the afflicted patients become disabled over the progression of this disease (6). RA is mediated predominantly by CD4$^+$ T cells overexpressing proinflammatory cytokines, particularly in the joints (7).

In order to test the efficacy of potential RA therapeutics and understand mechanisms of disease, the collagen-induced arthritis (CIA) model is often used (8). CIA is typically induced by immunizing rodents with bovine or chick type II collagen together with an adjuvant. This combination leads to immune attack of the host's native collagen involving components of both the innate and adaptive immune systems. Emphasis on regulating proinflammatory cytokines, particularly TNF-α, is key to minimizing disease since TNF-α can be detected in joints of RA patients (9, 10). Mouse CIA shares several clinical, histopathological and immunological features with human RA. Clinical features include erythema, edema, synovitis, pannus formation, and

$CD4^+$ T cell-mediated inflammation with extensive cartilage and bone damage, resulting in joint deformities (11–13). These similarities are commonly exploited to use CIA as a model for RA and as a tool to investigate novel approaches to prevent and treat RA. Current treatments focus on neutralizing TNF-α action via anti-TNF-α mAbs and TNF-α receptor antagonists (14, 15); however, such interventions have been problematic, making patients more susceptible to opportunistic infections (14–16). Hence, alternatives that can restore tolerance need to be sought.

In view of reducing autoimmunity, the use of probiotics can restore immune homeostasis to reduce autoimmunity (17–19). Historically, lactic acid bacteria (LAB) represented the core of probiotic-based interventions, although more recently nonpathogenic *E. coli* (20–22), attenuated *Salmonella* (23, 24), *Bifidobacterium spp.* (25), and some yeasts like *Saccharomyces boulardii* (26) also proved to be valuable tools as novel therapeutic and prophylactic interventions. Traditional molecular genetics, coupled with synthetic biology, provides an ample selection of promoters and terminators resulting in dynamic expression levels. Protein synthesis can be induced *in vitro* under nisin controlled expression (NICE), or use a promoter that is silent during *in vitro* culture, and only active *in vivo* subsequent infection of the host (27–29). LABs are considered ideal vectors for oral or mucosal delivery since they are inherently nonpathogenic, and they can survive the harsh conditions of the gastric environment. LABs are amenable to recombinant expression of passenger antigens (Ags) to stimulate immunity against a number of pathogens (30–32), to curb the effects of inflammatory bowel disease (33, 34), to control the proliferation of cancer cells (35), and to use for enzyme replacement therapy (36) among other applications (27, 37, 38). Currently, the only microbiota-based therapy that is FDA-approved and commercially available is fecal microbiota transplant (FMT) to treat *Clostridium difficile* infections. However, close to 200 microbiome-based therapeutics and diagnostics are currently in development (39).

The delivery of oral therapeutics represents a significant advantage of adapting LABs. In this context, we developed recombinant *Lactococcus lactis* (LL) for oral delivery to treat autoimmune disease (40). In a similar fashion, the studies described here focus on the expression of the immunosuppressive cytokine, IL-35. Oral administration of probiotic-based therapeutics is considered ideal because the gastrointestinal (GI) tract is home to T cells that can be stimulated to become Tregs and to seed other mucosal and systemic immune compartments. Another advantage of using genetically-modified (GM) probiotics is that these have been shown to be both effective and safe (37, 41–43). Our previous work has shown that an engineered LL derived from an industrial dairy strain can ferment commercial milk to a yogurt-like product, and when applied for treatment of CIA, can maintain the same therapeutic properties as when grown on a synthetic medium (40). IL-35 belongs to the IL-12 cytokine family [rev. in (44)]. This heterodimeric cytokine is composed of IL-12p35 and IL-27EBI3 and, in contrast to most members of the IL-12 family, has potent anti-inflammatory attributes. This property is mediated via IL-35 binding both IL-12Rβ2 chain and gp130, which results in specific triggering of STAT1 and STAT4 on T cells (45) and IL-12Rβ2 and IL-27Rα on B cells (46). IL-35 is immunosuppressive for a number of autoimmune disease models including CIA (47, 48), experimental autoimmune encephalomyelitis (49, 50), uveitis (46), type 1 diabetes (51), inflammatory bowel disease (IBD, and psoriasis (52).

CCR6 was previously shown to be expressed by Tregs (53), particularly those expressing RoRγt (54, 55). These Tregs have been shown involved in suppressing autoimmune diseases (56–58). $CCR6^+$ Tregs have been found more commonly associated with human Tregs (55, 59), but CCR6 has also been found to be induced in mice subjected to CIA (55).

Given its potency to treat various autoimmune diseases (46–52), we queried the effectiveness of live vector delivery of IL-35. To accomplish this objective, murine IL-35 was expressed in *L. lactis* subsp lactis IL1403 (LL-IL35), and tested for its ability to ameliorate CIA. Results show that LL-IL35 is highly effective in treating CIA via the stimulation of $CCR6^+$ and $CCR6^-$ Tregs producing IL-10 and suppressing the proinflammatory cytokines, IL-17 and IFN-γ.

MATERIALS AND METHODS

Bacterial Strain Engineering and Maintenance

Lactococcus lactis subsp. lactis IL1403 (IL1403) was grown on M17 plus 0.5% glucose (M17G). Microbiology work was performed according to NIH guidelines. Initial attempts to express IL-35 under the control of the constitutive p23 promoter yielded only rearranged, nonfunctional clones which confirmed the notion that IL-35 is difficult to express and to stabilize in a wide panel of hosts [our unpublished observations; (44, 48)]. To express IL-35, a synthetic gene codon-optimized for LL was designed in-house and then synthesized by Genscript (Piscathawa, NJ). The fragment contains an optimal Shine-Dalgarno sequence properly spaced from the ATG start codon, the usp45 secretion signal, the p35 coding region, a short flexible linker, the EBI3 coding region, and AgeI and SmaI sites at both ends. The fragment was excised with AgeI, gel-purified and cloned into pMSP3535H3 (53; a kind gift of Dr. DA Mills, University of California, Davis) yielding a construct named pBzMM150 (LL-IL35). Expression was achieved under the control of the nisin-inducible promoter borne on the vector.

Collagen-Induced Arthritis (CIA)

All the animal experiments described in the present study were conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal studies were conducted under protocols approved by Montana State University's and the University of Florida's Institutional Animal Care and Use Committee.

C57BL/6 males (B6; 8- to 10-weeks of age; Charles River Laboratories, Horsham, PA USA) were maintained at Montana State University Animal Resources Center or the University of Florida Animal Center Services. Groups of B6 males were

induced with CIA using 100 μg of chicken collagen II (CII; Chondrex, Redmond, WA USA) emulsified in complete Freund's adjuvant (CFA) and administered s.c. as previously described (48, 60, 61). To treat CIA, mice were first orally gavaged with sterile 50% saturated sodium bicarbonate solution to neutralize stomach acidity, followed by 5×10^8 CFUs of LL vector or LL-IL35, or vehicle only, sterile PBS. Two dosing regimens were tested, three doses administered on days 14, 21, and 28, and two doses given on days 18 and 25 post-CII challenge. Clinical scores were measured in a double-blind fashion after treatment, and mice were monitored to day 40. Each of the four limbs was evaluated using a scale of 0–3 (48, 60, 61): 0, no clinical signs; (1) mild redness of a paw or swelling of single digits; (2) significant swelling of ankle or wrist with erythema; (3) severe swelling and erythema of multiple joints; maximum score per mouse is 12.

Cytokine Elisa

$CD4^+$ T cells were cell-sorted by negative selection on magnetic beads (Invitrogen, Grand Island, NY USA) from axillary, popliteal, and inguinal lymph nodes (LNs) yielding purity >98%. Purified $CD4^+$ T cells (3×10^6/ml) were restimulated with 5 μg/ml plate-bound anti-CD3 mAb (eBioscience, San Diego, CA USA) plus 5 μg/ml of soluble anti-CD28 mAb (eBioscience) for 48–72 h at 37°C and 5% CO_2 similar to that previously described (48). Culture supernatants were collected for cytokine-specific ELISAs (48, 60, 61).

Flow Cytometry

Splenic and LN cells were stained with fluorochrome-labeled mAbs to CD4, CD39, Ly-6G, Ly-6C, CD11b, and Foxp3 (eBioscience, San Diego, CA USA), TGF-ß (R&D Systems, Minneapolis, MN USA), and fluorochrome-conjugated streptavidin (BD Pharmingen, San Jose, CA USA). For flow cytometry of Tregs, whole splenic and LN cells (5×10^6/ml culture) were restimulated overnight with 50 μg/ml of CII (T-Cell Proliferation; Chondrex). The next day, cells were stimulated with 25 ng/ml PMA and 1 μg/ml ionomycin for an additional 3 h. Cells were harvested, washed, stained and analyzed as previously described (48, 60, 61).

To measure inflammatory cells in the arthritic joints, isolated limb joints were digested with 2 mg/ml collagenase (*Clostridium histolyticum*,Type IV; Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C, and cell suspensions passed through a 70 μm cell strainer similar to that previously described (40, 62). Leukocytes were stained and analyzed by forward and side-scatter plots for $Ly\text{-}6G^+$ $Ly\text{-}6C^+$ $CD11b^+$ neutrophils.

Statistics

Mann-Whitney *U*-test was applied to statistically analyze clinical scores. The difference in arthritis incidence between experimental groups was checked with Fisher's exact probability test. One-way ANOVA was performed to analyze ELISA and flow cytometry results. Data were considered statistically significant, if *p*-value was < 0.05.

RESULTS AND DISCUSSION

RA is a chronic, systemic autoimmune disorder affecting millions of patients in the US. Treatment of this progressive, degenerative disease demands constant use of anti-inflammatory drugs and often immunosuppressive treatments that increase susceptibility to infections and neoplasia (4, 15, 16). Instead, intervention strategies that focus on redirecting or reeducating T cell responses to produce tolerance instead of inflammation have the potential of being a superior treatment for RA.

To address the void for such tolerance induction, we queried whether a probiotic LAB engineered to express the potent anti-inflammatory cytokine, IL-35 (**Figure 1A**), would diminish arthritis. Expression of IL-35 by LL-IL35 was detected by Western blot analysis using a rabbit polyclonal serum against an MBP-IL-35 fusion protein [produced in-house; (61)]. To test the therapeutic properties of LL-IL35, mice were challenged on day 0 with CII to induce CIA. Given its similarity, CIA is often exploited as an investigative tool to test novel strategies and therapeutics to prevent and treat RA. These mice were randomly divided into three groups for oral treatment: LL-IL35 (pBzMM150), LL vector (pMSP3535H3), or sterile PBS. Two treatment paradigms were tested: beginning intervention on day 14 resembling *Salmonella*-CFA/I treatment (60) with two additional doses on days 21 and 28 (**Figure 1B**) or beginning intervention at disease onset on day 18, followed by a second dose on day 25 (**Figure 1C**). Clinical scores were performed in double blind, and followed until day 39 post-induction. We generally do not see changes in disease severity beyond 39 days post-CII challenge.

Using the three-dose regimen, 50% of the LL-IL35-treated mice showed no symptoms and the remaining 50% developed minor symptoms as opposed to PBS- or LL vector-treated mice, who all developed severe arthritis by day 24 post-CII challenge. Notably, the severity of disease symptoms was significantly less ($p < 0.001$) in the LL-IL35-treated mice exhibiting an average clinical score of 1 in contrast to PBS- or LL vector-treated mice eventually achieving clinical scores of ∼9 (**Figure 1B**). To test if LL-IL35 is effective in arresting the disease after disease onset, additional groups of CIA mice were treated using a two-dose regimen on days 18 and 25. Under this treatment, 40% of the LL-IL35-treated mice developed CIA vs. 100% of those treated with PBS or LL vector (**Figure 1C**). Compared to the three-dose regimen, the disease severity was greater for the LL-IL35-treated CIA mice subjected to the two-dose regimen, although significantly less ($p < 0.05$) when compared to similarly treated PBS- or LL vector-dosed mice. These data show that LL-IL35 can effectively reduce the symptoms of arthritis and the incidence of disease via its immunosuppressive capacity. Moreover, these results show that fewer doses of IL-35 delivered by LL are needed to curtail arthritis when compared to treatment with soluble protein (47, 48, 51, 52).

Analysis of knee joints was performed to determine the extent of neutrophil infiltration. In agreement with these clinical findings, the LL-IL35-treated mice had markedly reduced $Ly\text{-}6G^+$ $CD11b^+$ cells (neutrophils) infiltrating the joints (**Figure 2A**) representing a 7- and 5-fold reduction compared to

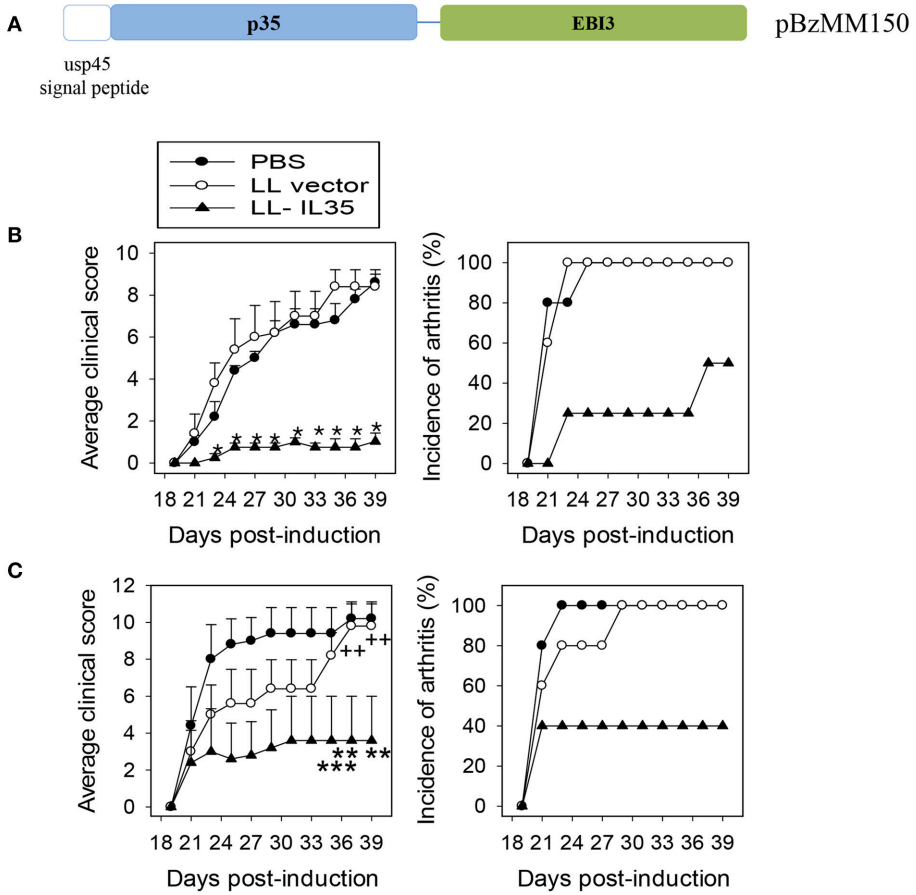

FIGURE 1 | IL-35 inhibits CIA progression following treatment with LL-IL35. **(A)** Schematic map of the synthetic DNA used to construct pBzMM150 for murine IL-35 expression of IL-35 in *L. lactis*. The synthetic insert encodes in order: the usp45 secretion peptide genetically fused in-frame to the p35 subunit, a short flexible linker, fused to the EBI3. The synthetic DNA also features an optimal Shine-Dalgarno (SD) sequence at the optimal distance from the ATG initiation codon, in addition to the SD sequence present in the vector. The nisin-inducible promoter and a transcription terminator are borne on the expression vector pMSP3535H3. **(B,C)** CIA was induced in groups of C57BL/6 males with chick CII emulsified in complete Freund's adjuvant. Two regimens were tested: orally treated with 5×10^8 CFUs of LL vector or LL-IL35 or sterile PBS on **(B)** 14, 21, and 28 days or **(C)** 18 and 25 days post-induction. Average clinical score per treatment group (left panels) represents severity of the disease, and incidence of arthritis depicts percent mice with affected joints in each treatment group (right panels). The sum of 10 mice/group is shown: **(B)** $^{*}p <$ 0.001 vs. PBS-dosed or LL vector-treated mice, and **(C)** $^{**}p < 0.02$, $^{***}p < 0.05$ vs. PBS-dosed mice and $^{++}p < 0.02$ vs. LL vector-treated mice .

PBS-dosed or LL vector-treated groups, respectively (**Figure 2B**). Hence, IL-35 can reduce inflammation of the joints in CIA-challenged mice.

To investigate the possible mechanism of protection conferred by LL-IL35, $CD4^+$ T cells purified from draining LNs were anti-CD3 + anti-CD28-restimulated and analyzed for cytokine production. These $CD4^+$ T cells were obtained from mice dosed three times with PBS, LL vector, or LL-IL35 as described in **Figure 1B**. IFN-γ levels remained elevated between PBS-dosed and LL vector-treated mice, in contrast to LL-IL35-treated mice showing 3- to 3.7-fold reduction ($p < 0.001$; **Figure 3A**). Concomitantly, IL-17 levels were significantly less ($p < 0.001$) by 3.7- and 8.5-fold for LL vector and LL-IL35-treated groups, respectively, relative to PBS-dosed mice (**Figure 3B**). Moreover, treatment with LL-IL35 significantly reduced IL-17 by 2.3-fold compared to LL vector-treated mice ($p < 0.01$). Minimal stimulation of IL-10 was detected in the restimulated $CD4^+$ T cells from the PBS-dosed mice (**Figure 3C**). In contrast, $CD4^+$ T cells from LL vector- and LL-IL35-treated groups showed significantly increased IL-10 production ($p < 0.05$) by 1.8- and 2.5-fold, respectively. The difference between the LL vector- and LL-IL35-treated groups was significant ($p < 0.05$; **Figure 3C**) as well. The stimulation of IL-10 by LL vectors has been reported by others (56, 57). However, IL-10 induced by the LL vector-treated group was insufficient to suppress disease progression (**Figure 1**) and IL-17 production (**Figure 3B**). IL-10's importance for suppressing CIA was previously demonstrated in $IL\text{-}10^{-/-}$ mice with CIA being refractive to IL-35 treatment (48), supporting the notion here of IL-10's relevance to CIA mice treated with LL-IL35. IL-35 has also been shown to stimulate IL-10 production (40, 47–49).

We have found that $CD39^+$ $CD4^+$ T cells are the primary Tregs responsible for resolving CIA (48, 60, 61). CD39 is an ectonucleoside triphosphate diphosphohydrolase-1which

hydrolyzes ATP into AMP, thus quenching inflammatory signaling by extracellular ATP (58, 63). We also showed that CD25$^+$ Tregs remained a subset of CD39$^+$ CD4$^+$ T cells, and that CD39 encompassed all of the Treg subsets (60). In fact, CD39$^+$ Tregs were protective against CIA (40, 60). These Tregs are composed of two subsets, Foxp3$^+$ and Foxp3$^-$, and are interchangeable (60). Analysis of induction of CD39$^+$ Tregs by the LL vector revealed no increase in the percentage of these Tregs in CIA mice (40), and CIA had only a modest impact upon their induction (40, 61).

To examine the types of Tregs induced by LL-IL35 treatment, whole splenic and draining LN lymphocytes were cultured overnight with CII, and then pulsed with PMA + ionomycin to ascertain the type of Tregs induced in PBS-dosed and LL-IL35-treated mice. Lymphocytes were then stained for CD39, Foxp3, and CCR6 to identify the Treg subsets. Since CCR6 has been shown to be expressed by Tregs (53–55), we queried whether such Tregs may be induced as a consequence of IL-35 treatment. CD39$^+$ CD4$^+$ T cells were evaluated for expression of Foxp3 and CCR6 (**Figures 4A,B**). Upon examination of splenic Tregs derived from PBS-dosed mice compared to those present in LL-IL35-treated mice, a modest increase ($p < 0.05$) in the frequency, but not the total number of CCR6$^+$ Foxp3$^+$ CD39$^+$ CD4$^+$ T cells, was observed (**Figures 4C,G**). A modest difference ($p \leq 0.01$) was also observed in the frequency and total number of splenic CCR6$^+$ Foxp3$^-$ CD39$^+$ CD4$^+$ T cells when compared to the PBS-dosed mice (**Figures 4E,I**). However, when similar analysis was performed for Tregs obtained from the draining LNs, a 2.2-fold increase in the frequency ($p < 0.001$) of CCR6$^+$ Foxp3$^+$ CD39$^+$ CD4$^+$ T cells was stimulated by LL-IL35 treatment compared to those present in PBS-dosed CIA mice (**Figure 4D**). The total number of these LN Tregs was also significantly ($p \leq 0.01$) increased by 2.7-fold (**Figure 4H**). Subsequent analysis was performed on LN CCR6$^+$ Foxp3$^-$ CD39$^+$ CD4$^+$ T cells, and both the frequency and total number increased significantly by 2.9- ($p \leq 0.01$) and 5.1-fold ($p < 0.001$), respectively (**Figures 4F,J**). These studies demonstrate that indeed CCR6$^+$ Tregs are induced by IL-35 treatment of CIA mice.

Additional analyses were performed on both CCR6$^-$ Foxp3$^+$ and CCR6$^-$ Foxp3$^-$ CD39$^+$ CD4$^+$ T cells (**Figures 4K–R**). Examination of the splenic CCR6$^-$ Foxp3$^+$ CD39$^+$ CD4$^+$ T

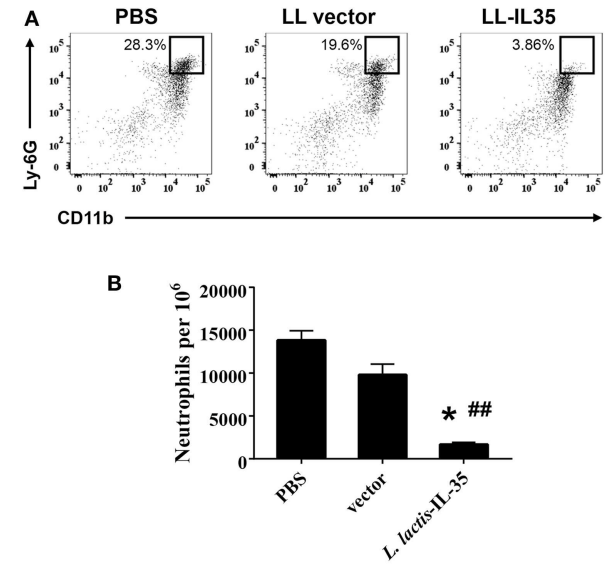

FIGURE 2 | Oral LL-IL35 confers protection against CIA via reduction of neutrophil influx into joints. Reduced clinical scores and disease incidence described in **Figure 1B** are attributed to reduced neutrophil infiltration into the joints of LL-IL35-treated mice when compared to PBS-dosed and LL vector-treated mice. **(A)** Cell suspensions were analyzed by flow cytometry for Ly-6G$^+$ CD11b$^+$ neutrophils, and **(B)** quantified per 10^6 cells. The depicted plots are representative of 5 mice/group; $^*p < 0.001$ vs. PBS-dosed mice; $^{\#\#}p < 0.005$ vs. LL vector-treated mice.

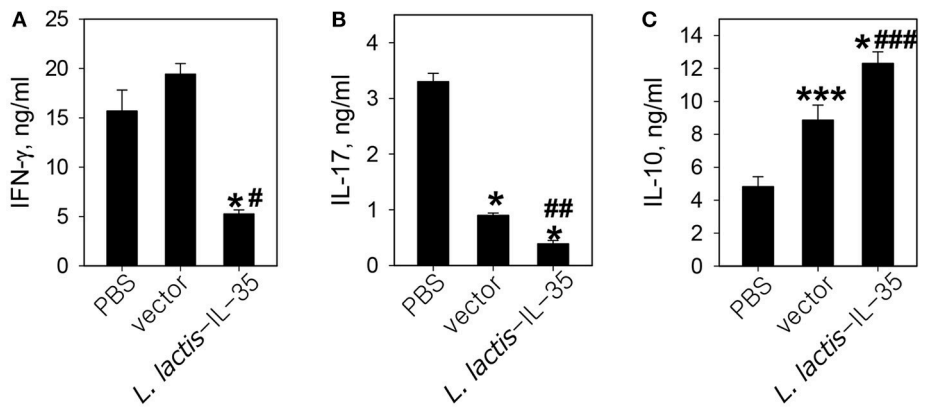

FIGURE 3 | LL-IL35 reduces proinflammatory cytokines, IFN-γ and IL-17, with a concomitant increase in anti-inflammatory IL-10 production. CD4$^+$ T cells were purified from PBS- or LL-IL35-dosed mice that received three treatments as described in **Figure 1B**. The LN CD4$^+$ T cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 for 48–72 h, and collected supernatants were analyzed for **(A)** IFN-γ, **(B)** IL-17, and **(C)** IL-10 production. Depicted are the means ± SD of triplicate cultures as assessed by cytokine-specific ELISA; $^*p < 0.001$, $^{***}p < 0.05$ vs. PBS-dosed mice; $^{\#}p < 0.001$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.05$ vs. LL vector-treated mice.

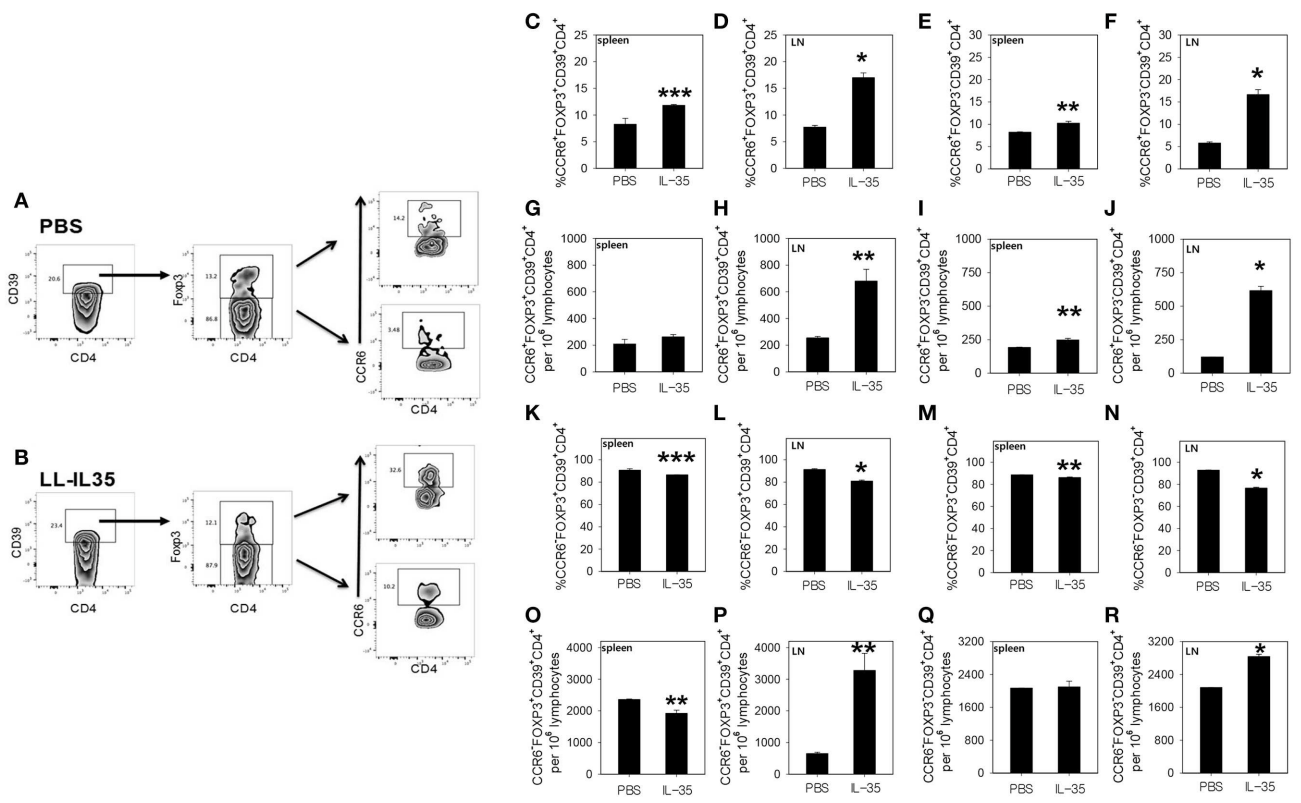

FIGURE 4 | LL-IL35 induces CCR6$^+$ and CCR6$^-$ CD39$^+$ CD4$^+$ T cells in CIA mice. At the termination of the study, whole splenic and LN lymphocytes were restimulated with 50 μg/ml CII overnight, and then subjected to a short-term of PMA + ionomycin. LN CD39$^+$ CD4$^+$ T cells from **(A)** PBS-dosed and **(B)** LL-IL35-treated mice were gated on Foxp3$^+$ and Foxp3$^-$ cells, and analyzed for **(C–R)** for CCR6 expression by **(C,E,G,I,K,M,O,Q)** splenic and **(D,F,H,J,L,N,P,R)** LN lymphocytes. **(C,D)** Frequency of CCR6$^+$ Foxp3$^+$ and **(E,F)** CCR6$^+$ Foxp3$^-$ and absolute **(G,H)** CCR6$^+$ Foxp3$^+$ and **(I,J)** CCR6$^+$ Foxp3$^-$ T cells are shown. **(K,L)** Frequency of CCR6$^-$ Foxp3$^+$ and **(M,N)** CCR6$^-$ Foxp3$^-$ and absolute **(O,P)** CCR6$^-$ Foxp3$^+$ and **(Q,R)** CCR6$^-$ Foxp3$^-$ T cells are also shown. Depicted are the means ± SEM of 5 mice/group; $^*p < 0.001$, $^{**}p \leq 0.010$, and $^{***}p < 0.05$ compared with PBS-dosed mice.

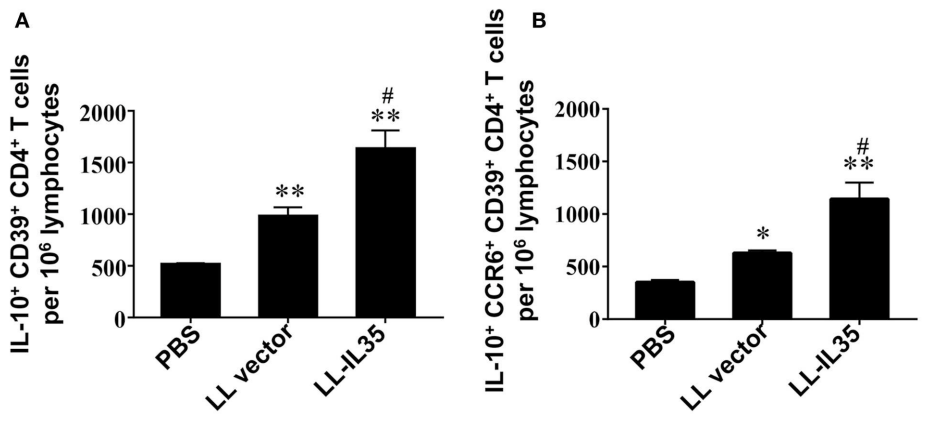

FIGURE 5 | LL-IL35 stimulates IL-10 production by CD39$^+$ Tregs and CCR6$^+$ CD39$^+$ Tregs. CIA mice treated with PBS, LL vector, or LL-IL35 as described in **Figure 4**. Intracellular IL-10 was measured for **(A)** CD39$^+$ CD4$^+$ and **(B)** CCR6$^+$ CD39$^+$ CD4$^+$ T cells. Depicted are the means ± SEM of 5 mice/group; $^*p < 0.001$, $^{**}p < 0.01$ vs. PBS-dosed mice; $^{\#}p<0.01$ vs. LL vector-treated mice.

cells revealed that both the frequency and total number were modestly and significantly ($p < 0.05$) reduced for the LL-IL35-treated mice (**Figures 4K,O**). The frequency of splenic CCR6$^-$ Foxp3$^-$ CD39$^+$ CD4$^+$ T cells was slightly and significantly ($p \leq 0.01$) reduced (**Figure 4M**), but the total number of these CD39$^+$ CD4$^+$ T cells showed no difference between PBS-dosed

and LL-IL35-treated CIA mice (**Figure 4Q**). Similar analysis was also performed for the LN CCR6$^-$ Foxp3$^+$ and CCR6$^-$ Foxp3$^-$ CD39$^+$ CD4$^+$ T cells from the same treated CIA mice. While a slight reduction in the frequency of LN CCR6$^-$ Foxp3$^+$ CD39$^+$ CD4$^+$ T cells was observed for LL-IL35-treated mice (**Figure 4L**), the total number of CCR6$^-$ Tregs was significantly ($p \leq 0.01$) elevated by 5-fold (**Figure 4P**). Examination of the frequency of LN CCR6$^-$ Foxp3$^-$ CD39$^+$ CD4$^+$ T cells also showed a modest, but significant ($p < 0.001$) reduction in LL-IL35-treated mice relative to PBS-dosed mice (**Figure 4N**), but the total number of these LN T cells was significantly ($p < 0.001$) enhanced by 36% (**Figure 4R**). Hence, these analyses demonstrate that IL-35 treatment stimulates diverse subsets of Tregs including both CCR6$^+$ and CCR6$^-$ Tregs. Future studies will need to consider the longevity of these subsets for protection against CIA.

Inquiring into the activity of these LN CD39$^+$ Tregs, analysis for IL-10 production was performed (**Figure 5**). Intracellular IL-10 measurements were conducted first for all CD39$^+$ CD4$^+$ T cells (both Foxp3$^+$ and Foxp3$^-$). The LL-IL35-treated mice showed 3.2- and 1.7-fold more IL-10-producing cells ($p \leq 0.01$) than PBS-dosed and LL vector-treated CIA mice, respectively (**Figure 5A**). LL vector-treated mice showed 1.9-fold increase in the number of IL-10-producing CD39$^+$ CD4$^+$ T cells compared to PBS-dosed mice ($p \leq 0.01$; **Figure 5A**). Examination of IL-10$^+$ CCR6$^+$ CD39$^+$ CD4$^+$ T cells (both Foxp3$^+$ and Foxp3$^-$) revealed that two-thirds of the total IL-10-producing cells induced by LL-IL35 treatment of CIA mice were derived from the CCR6$^+$ subset (**Figure 5B**). The CCR6$^+$ CD39$^+$ CD4$^+$ T cells induced with LL-IL35 resulted in significant 3.2- and 1.8-fold increase in IL-10-producing cells than those present in PBS-dosed ($p \leq 0.01$) and LL vector-treated CIA mice ($p < 0.01$), respectively. LL vector-treated mice showed 1.8-fold increase in the number of IL-10-producing CCR6$^+$ CD39$^+$ CD4$^+$ T cells compared to PBS-dosed mice ($p \leq 0.01$; **Figure 5B**). These findings suggest that indeed both Foxp3$^+$ and Foxp3$^-$ CCR6$^+$ CD39$^+$ Tregs are the predominant source of IL-10, thus contributing to the amelioration of CIA subsequent LL-IL35 treatment. Such finding may mimic what is evident with human peripheral blood CCR6$^+$ CD39$^+$ Tregs (64) and CCR6$^+$ Tregs found in patients with glomerulonephritis (65).

The data presented demonstrate the potency of IL-35 as an anti-inflammatory therapeutic. Moreover, this investigation further supports the multifaceted benefits of adapting recombinant *L. lactis* as a vector to deliver therapeutic doses of IL-35. In fact, previous studies by us (48) or others, using IL-35 to treat type 1 diabetes model (51), IBD (52), or psoriasis (52), required daily treatments with recombinant protein to control disease. In contrast, only two or three oral doses of LL-IL35 were sufficient to prevent the onset or stop CIA progression. Oral dosing has the substantial advantage of being less invasive circumventing the need for injections. LL-derived IL-35 eliminates the labor-intensive efforts needed to produce and purify the recombinant protein, dramatically reducing the cost of manufacturing this therapeutic. Moreover, IL-35 is a dimeric protein which adds to the difficulty and cost to generate. The *L. lactis* used for this study is a lab-adapted recombinant strain, originally derived from an industrial dairy strain capable of fermenting milk into a product that has the same textural and olfactory properties of yogurt (40). We previously have demonstrated that the curative properties of our recombinant *L. lactis* are maintained when grown on a synthetic medium or used to ferment milk into a yogurt-like product (40). These attributes make *L. lactis* an ideal tolerogen delivery platform for the treatment of autoimmune diseases.

AUTHOR CONTRIBUTIONS

MM, IK, and DP: conceptualization. MM, IK, CH, and DP: formal analysis, investigation, methodology, validation, visualization, and writing. DP: funding acquisition. All authors approved the final version to be published and agreed to the content and all aspects of the work ensuring the accuracy or integrity of the work are appropriately investigated and presented.

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An Insight Into the Intestinal Web of Mucosal Immunity, Microbiota, and Diet in Inflammation

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The rising global incidence of autoimmune and inflammatory conditions can be attributed to changes in the large portion of the immune system that belongs to our gastrointestinal tract (GI). The intestinal immune system serves as a gatekeeper to prevent pathogenic invasions and to preserve a healthier gut microbiota. The gut microbiota has been increasingly studied as a fundamental contributor to the state of health and disease. From food fermentation, the gut microbiota releases metabolites or short chain fatty acids (SCFAs), which have anti-inflammatory properties and preserve gut homeostasis. Immune responses against food and microbial antigens can cause inflammatory disorders such as inflammatory bowel disease (IBD) and celiac disease. As such, many autoimmune and inflammatory diseases also have a "gut origin". A large body of evidence in recent years by ourselves and others has uncovered the link between the immune system and the SCFAs in specific diseases such as autoimmune type 1 diabetes (T1D), obesity and type 2 diabetes (T2D), cardiovascular disease, infections, allergies, asthma, and IBD. Thus, the power of these three gut dynamic components—the mucosal immunity, the microbiota, and diet—can be harnessed in tandem for the prevention and treatment of many inflammatory and infectious diseases.

Keywords: mucosal immunity, gut microbiota, SCFAs, diet, IELs, autoimmunity, diabetes, infections

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INTRODUCTION

Considered the largest immunological organ in our body, the gut is in constant contact with food antigens, commensal microbiota, and foreign pathogens. As such, the gut has evolved extremely dynamic anatomy capable of regulating innate and adaptive immune responses to manage these interfaces effectively (1). In a homeostatic environment, there is a mutualistic relationship between the host and the microbiota. Keeping the delicate balance between mucosal immunity and microbiota (commensal bacteria) is a complex process. A disruption to the gut microbiota ecology, also known as dysbiosis, can lead or contribute to disease (2–4). While the host provides a nutrient-rich environment, the microbiota, in turn, provides the host with an increased capacity to harvest nutrients from food and boost the host immune system. The mucosal immune system, as a physical barrier, prevents pathogenic microorganisms and immunogenic components from traversing from the mucosae into the internal environment of the host. At the same time, this tightly regulated system has the important task of inducing tolerance to antigens (1). Here, we review the complex interplay between intestinal immunity, gut microbiota, and diet in inflammatory diseases (**Table 1**), such as T1D and IBD (22), and infection in humans and animal models.

TABLE 1 | Diet, intestinal immunity and microbiota in diseases.

Disease	Involvement of intestinal immunity and the gut microbiota	References	
		Animal models	Humans
Type 1 diabetes (T1D)	• Limited or altered microbiota diversity • Increased gut permeability • Intestinal immune system in a constant state of pro-inflammation	(5–12)	(13–21)
Inflammatory bowel disease (IBD)	• Reduction of butyrate-producing bacteria/butyrate production	(22, 23)	(24, 25)
	• Tryptophan-rich diet and L.reuteri induced differentiation of $TCR\alpha\beta^+CD4^+CD8\alpha\alpha^+$ IELs associated with protection	(26–29)	
	• Pathogenic role for $TCR\gamma\delta^+$ IELs in colitis	(30, 31)	
Infections	• Absence of commensal microbiota regulated $CD8\alpha\beta^+$ IELs associated with protection against bacterial and viral infections	(32–35)	
	• Crosstalk between IECs and $TCR\gamma\delta^+$ IELs within the epithelium prevented pathogen invasion	(36–40)	

DYNAMIC TRIO: INTESTINAL IMMUNE SYSTEM, GUT MICROBIOTA, AND DIET

A network of intestinal immune cells resides within the epithelium and consists of three distinct lymphoid structures. This physical and biochemical barrier to commensal and pathogenic microorganisms is formed by the epithelium layer, the lamina propria layer (LPL) and the gut-associated lymphoid tissues (GALT). The GALT comprises the mesenteric lymph nodes (MLNs), the Peyer's patches (PPs) and isolated lymphoid follicles (ILFs) (1). Intestinal epithelial cells (IECs) have fundamental immuno-regulatory functions. For example, trans-presentation of IL-15 by IECs drives the development and differentiation of immuno-regulatory CD8αα intraepithelial lymphocytes (IELs) (41). Furthermore, overexpression of epithelial IL-15 is essential for the motility and localization of protective γδ IELs into the epithelium of small intestine (42). The IECs also express anti-inflammatory cytokines such as IL-10, which likely contributes to tolerance to commensal bacteria and epithelial integrity through epithelial-macrophage crosstalk (43). Besides that, epithelial cells are reinforced by tight junctions to form an interconnected network that acts as the main mechanical barrier of the intestinal mucosal surface. Tight junctions, such as zonulins, occludins and claudins, are essential for border control and are strictly regulated in response to various signals to preserve cellular polarity (44). Specialized IECs such as goblet and Paneth cells reinforce barrier function by the secretion of antimicrobial peptides (AMPs) by enterocytes (45). These AMPs, such as C-type lectin regenerating islet-derived protein IIIγ (REGIIIγ) and defensins, disrupt surface membranes of bacteria; thus enabling a broad regulation of commensal and pathogenic bacteria (46–48).

The intestinal innate immune system mediates the symbiotic relationship between the host and the gut microbiota. Myeloid differentiation primary response gene 88 (MyD88) is a central adaptor molecule for the majority of Toll-like receptors (TLRs) involved in pathogen recognition. To preserve symbiosis, IECs need to recognize pathogenic molecule patterns. Previous data support MyD88 playing a key role in regulating the immune system by shaping the gut microbiota (6). Furthermore, IEC MyD88 has been suggested to act as a primary sensor involved in the cross-talk between nutrients, the gut microbiota and the host during diet-induced obesity (DIO) (49). The specific deletion of IEC MyD88 partially protected against DIO, diabetes and inflammation. Previously, Ley et al. (50) discovered that genetically obese mice have a 50% reduction in the abundance of Bacteroidetes and a proportional increase in Firmicutes. In another study, the authors performed microbiota transplantation experiments and demonstrated that the obese microbiota had an increased capacity to harvest energy from the diet (51). Additionally, IECs are also involved in the adaptive immune regulation of gut homeostasis mediated by IgA (52). Another type of specialized IECs is concentrated in the follicle-associated epithelium which overlays the luminal surface of lymphoid structures including PPs and ILFs. These microfold cells or M cells facilitate the sampling of luminal antigens and intact microorganisms for presentation to the intestinal immune system (53, 54). While both M cells and goblet cells have been shown to be involved in antigen delivery to the LPL, their functional importance to and influence on the regulation of pathogen responses or immune tolerance remain incompletely identified.

The connections between the gut microbiota and the mucosal immunity are vastly influenced by dietary fiber and microbial SCFAs. Fermentation of different foods, such as indigestible polysaccharides by the gut micrbiota, produces SCFA acetate, propionate and butyrate (55, 56). For instance, *Bacteroides thetaiotaomicron*, an acetate producer, upregulated KLF4, which is involved in goblet cell differentiation and increased expression of mucin-related genes. *Faecalibacterium prausnitzii*, an acetate consumer and butyrate producer, attenuated the effects of *B.*

thetaiotaomicron to prevent mucus overproduction, necessary for a healthier epithelium structure and composition (57). The gut epithelium is covered by a mucus layer and its integrity is vital to prevent microbe invasions and infections (1). As elucidated by Desai et al. (58), a chronic or intermittent dietary fiber deficiency caused the gut microbiota to resort to using host-secreted mucus glycoproteins as a nutrient source, leading to erosion of the colonic mucus barrier. The goblet cells secrete heavily glycosylated proteins called mucins into the intestinal lumen as a first line of defense against microbial invasion (45). Mucin 2 (MUC2), the most abundant of these mucins, is pivotal in shaping the intestinal mucus layers at epithelial surfaces. Mice deficient in MUC2 experienced fatal colitis when infected with *Citrobacter rodentium* (59). It is striking to observe that simple deprivation of dietary fiber in wild-type mice for either the whole duration or intermittently in the 40-day study period was sufficient to imitate the phenotype in MUC2-deficient mice. Furthermore, a prolonged lack of dietary fiber is associated with increased abundance of mucin-degrading bacteria such as *Akkermansia muciniphila* (58). Consistent with this, feeding mice a Western diet with very low fiber content has been shown to strongly affect gut microbiota composition, reduce mucus growth rate and increase penetrability of the inner colonic mucus layer (60). Given that fiber is the primary source of energy for the gut microbiota, the results ascribed to the above studies point to the critical effect of microbial SCFAs. As such, *Bifidobacterium* strains have long been used as probiotics, with several beneficial effects attributed in part to the increased production of acetate (61). For example, colonization of germ-free (GF) mice with bifidobacteria prevented translocation of enteropathogenic *Escherichia coli* O157 (61). Although supplementation of 1% inulin (a prebiotic with bifidogenic effect) or *Bifidobacterium longum* prevented mucus defects, it was insufficient to improve metabolic parameters of obese animals. Contrastingly, a high intake of 20% inulin reduced microbiota encroachment into the mucosa, prevented colonic atrophy and increased intestinal epithelial proliferation, thereby protecting mice against DIO (62).

IELS: FRIEND OR FOE IN DISEASE?

To sustain gut barrier integrity, the mucosal immune system is prepared to provide extra protection against infection and inflammation. Commonly referred to as intestinal intraepithelial lymphocytes (IELs) (63), most IELs express the CD8αα homodimer and approximately 90% express T cell receptors (TCRs) (64) and can be classified as induced (conventional) or natural (unconventional). Induced IELs are derived from peripheral T cells that home to the intestinal epithelium after encountering antigens in gut-associated lymphoid tissues (GALT). Induced IELs can be divided into subsets that express either CD4 or CD8αβ, upon entering the intestinal epithelium (63). Induced IELs typically exhibit an effector-memory-like phenotype and are thought to have a protective role against pathogenic invasions (63). A fraction of the induced $TCR\alpha\beta^+CD4^+$ T cells migrate to the intestinal epithelium upon activation in GALT where they can act as effector or tissue-resident memory T cells. While $CD4^+$ T cells have been traditionally thought to lose the CD8 chains after thymic development, some studies have reported the existence of $TCR\alpha\beta^+CD4^+$ IELs that also express CD8αα both in mice (65, 66) and humans (67), which do not possess every quality of mature $CD8^+$ T cells. In order to gain expression of CD8αα, mature $CD4^+$ T cells need to reactivate CD8-lineage genes by terminating expression of T helper master regulator (ThPOK), while concurrently upregulating expression of the transcription factors Runx3 (68) and T-bet (69). External stimuli within the intestinal epithelium, for example, TGF-β, retinoic acid, IFN-γ, and IL-27, serve as cues for the re-differentiation of $CD4^+$ T cells (69–71).

Recent data confirm the gut microbiota as yet another central factor in the generation of $TCR\alpha\beta^+CD4^+CD8\alpha\alpha^+$ IELs (26). For example, a tryptophan metabolizing commensal microorganism called *Lactobacillus reuteri* has been revealed to activate the transcription factor aryl hydrocarbon receptor (AhR), important for immune regulation in mucosal inflammation. Tryptophan is mostly found in foods such as oats, dairy products, seeds and, nuts. Mice without *L. reuteri* have significantly low numbers of $TCR\alpha\beta^+CD4^+CD8\alpha\alpha^+$ IELs, and reconstitution of *L. reuteri* in these mice, coupled with a diet rich in tryptophan, was adequate to induce $TCR\alpha\beta^+CD4^+CD8\alpha\alpha^+$ IELs differentiation (26). This IEL subset has an immune regulatory effect in the prevention of IBD (27, 28). An elegant study by Sujino and colleagues further demonstrated a microbiota-dependent conversion of LPL $Foxp3^+$ T_{regs} into $TCR\alpha\beta^+CD4^+CD8\alpha\alpha^+$ IELs upon migration to the epithelium to control intestinal inflammation (29). $TCR\alpha\beta^+CD8\alpha\beta^+$ IELs are another type of induced IELs that make up 10-15% of the IEL population in mice (63) and 70–80% in humans (67). The majority of this subset is derived from peripherally activated $CD8^+$ T cells that eventually migrate to the intestinal epithelium (72) and remain there. These cells have properties distinct to antigen-experienced $CD8^+$ T cells found in peripheral lymphoid organs (73) in the expression of activation markers and cytokine secretion. The expression of CD8αα, which decreases the sensitivity of the TCR to antigens, is another main factor differentiating peripheral and intestinal $CD8^+$ T cells (74). Presently, the role of $TCR\alpha\beta^+CD8\alpha\beta^+$ IELs in mucosal immunity is still unclear. Adoptive transfer studies have demonstrated a protective function of antigen-specific $CD8\alpha\beta^+$ IELs in mouse models of bacterial and viral infections (32, 33). Although the $CD8\alpha\beta^+$ IELs are strongly cytotoxic, they typically encounter commensal bacteria rather than invasive pathogenic microorganisms. Many studies comparing conventional, GF and antibiotic-treated mice and attempting to explore the connection between commensal bacteria and $CD8\alpha\beta^+$ IELs have found a positive relationship, where the former may be involved in regulating the number of $CD8\alpha\beta^+$ IELs in the intestinal epithelium (34, 35). Whilst that may be true, the mechanism behind this phenomenon is unknown. A recent study by Chen et al. (75) identified a series of genes that encode antimicrobial peptides, especially α-defensins, in commensal-dependent $CD8\alpha\beta^+$ IELs using microarray analysis. Applying 16S rDNA sequencing techniques in their microbiota-transplantation experiment, the authors showed that depleting

the *Bifidobacterium* species using antibiotics increased the number of CD8αβ$^+$ IELs. They further demonstrated that the IL-15/IL-15R complex influenced by commensal bacteria enhanced the antimicrobial activity of CD8αβ$^+$ IELs *in vitro*. However, the mechanistic link showing how a specific bacterial species or particular microbial metabolites could influence these IEL subsets remains unexplored.

While the gut immune system can replenish itself, interaction with the thymus is also essential to support gut homeostasis. Natural IELs such as TCRγδ$^+$ cells migrate directly to the intestinal epithelium after development in the thymus (63). However, there is evidence to suggest the extrathymic origin of many TCRγδ$^+$ IELs (76). TCRγδ$^+$ cells are not only limited to the intestines, but have also been found in other mucosal sites such as the skin and uterus where they contribute to the maintenance of barrier integrity (77). These cells have been shown to have dynamic migratory patterns within the intestinal epithelium mediated by occludin, a tight junction protein (36, 37). We found that increased expression of occludin in the colon of non-obese diabetic (NOD) mice fed with a high butyrate-yielding diet was associated with protection against autoimmune diabetes (5). Remarkably, we found butyrate increased colonic TCRγδ$^+$ IELs correlated to increased expression of IL-15 in the thymus (Y. Yap, J. L. Richards & E. Mariño, unpublished data). Epithelial IL-15 is essential for the migration of TCRγδ$^+$ IELs into the intestinal epithelium (42). Additional *in vitro* analyses further supported the importance of epithelial IL-15 and IL-2Rβ in TCRγδ$^+$ IEL motility within the intestinal mucosa. Impaired IL-2Rβ signaling and overproduction of IL-15 locally in the lamina propria resulted in acute pathogenic *Salmonella typhimurium* invasion and enhanced bacterial translocation (42). A fundamental role of TCRγδ$^+$ IELs is to protect the intestinal epithelium against pathogenic microorganisms and inflammation. Crucial in the mucosal immune response against resident intestinal bacteria, TCRγδ$^+$ IELs produce antimicrobial effectors such as RegIIIγ when in contact with resident bacterial pathobiont within the IECs (38). This antibacterial response is dependent on MyD88 signaling in IECs, which further highlights the microbe-dependent cues for TCRγδ$^+$ IEL homeostasis. This crosstalk between IECs and TCRγδ$^+$ IELs is not only crucial for intestinal homeostasis with resident microbiota but also necessary in the protection against pathogens, such as *S. typhimurium* (38), *Toxoplasma gondii* (39) or the parasitic *Nippostrongylus brasiliensis* (40). The function of TCRγδ$^+$ IELs may differ between the small intestine and colon and may be regulated by different mechanisms depending on the presence of injury to mucosal tissues (38, 78, 79). To protect the epithelium against inflammatory damage during infection, TCRγδ$^+$ IELs produce a variety of factors such as TGF-β and keratinocyte growth factor (KGF) to promote healing and integrity of the intestinal epithelium (80).

The cytotoxic and repairing properties of TCRγδ$^+$ IELs is evident in infections by fighting off early microbial invasion and limiting excessive tissue damage at the end of the infection phase. In experimental models of colitis, TCRγδ$^+$ IELs appear to have diverging roles. Some studies have demonstrated a pathogenic role for these cells in colitis induction (30, 31). Interestingly, as the disease progressed to its later stages, TCRγδ$^+$ IELs play a protective role against inflammation-induced epithelium damage (81, 82). Mice lacking cytosolic sensor for microbial DNA, STING, are highly susceptible to colitis and acute infection (83). Exacerbated colonic inflammation correlated with a significantly higher frequency of natural TCRγδ$^+$ IELs, decreased induced TCRαβ$^+$CD8αβ$^+$ and TCRαβ$^+$CD8αα$^+$ IELs and impaired development and function of Foxp3$^+$ T_{regs}. As such, these findings strongly suggest the highly variable impact of the microbiota on the frequency and function of intestinal IELs in infection settings. Intestinal IELs have largely been studied in disease settings where they are involved in mechanistic links with the gut microbiota. In fact, mice were protected from ischemic stroke when intestinal homeostasis was restored (84). Mice induced with acute brain injury presented an altered gut microbiota, which promote the trafficking of effector IL-17-producing TCRγδ$^+$ cells from the small intestine to brain. Protection against ischemic stroke was correlated with the suppression of effector TCRγδ$^+$ cells (84). In autoimmune diabetes, TCRγδ$^+$ cells, depending on their origin and condition, can either protect (85–87) or exacerbate (7) the disease, once again highlighting the dual role of these cells in disease.

DIETARY FACTORS IN ACTION

Environmental factors are dominant over host genetics in shaping the human gut microbiota (88). As we have shown, diet is one environmental factor that highly influences the gut microbiota and the immune system (5, 22, 89, 90). Diet is composed of different macro- and micronutrients, and natural sources of fiber are necessary for the production of SCFAs (56). Conversely, changes in our dietary habits to include highly processed and refined foods, additives such as emulsifiers and preservatives, can negatively impact on gut microbial composition, which may adversely affect our health (91–94). Acetate, propionate and butyrate contribute to a healthy gut microbiota and for a mature and developed immune system (55). We have shown in a mouse model of colitis, the SCFAs, acetate and butyrate protected against intestinal epithelial damage in DSS-induced colitis (22). High fiber feeding promoted the release of SCFAs, which led to inflammasome activation through GPR43 and GPR109A (22). This event led to the production and secretion of IL-18, a cytokine involved in repairing intestinal epithelial integrity, and homeostasis. The high levels of IL-18 and protection from DSS colitis were associated with a significant increase in *Bacteroidetes*, which were predominantly found in mice fed a high fiber diet. Conversely, species that were considerably low or absent in mice fed a zero-fiber diet were bacteria from the genus *Prevotella*, known to contain a set of bacterial genes for cellulose and xylan hydrolysis. This is consistent with findings in children from rural Burkina Faso study, whose microbial diversity is associated with the extraction of valuable nutrition from fibrous plant foods (95, 96).

SCFAs also regulate the immune cells that reside in the LPL. As such tolerogenic mucosal CD103$^+$ dendritic cells (DC) conferred protection in a murine food allergy model through high fiber

feeding. In this study, we found a high fiber diet increased the release of acetate and butyrate associated with enhanced oral tolerance and retinal dehydrogenase (RALDH) activity (90). Furthermore, protection from food allergy was dependent on vitamin A found in the diet. Accordingly, mice lacking GPR43 or GPR109A (metabolite sensing receptors for acetate and butyrate) displayed exacerbated food allergy and reduced $CD103^+$ DCs. Likewise, butyrate produced by commensal bacteria promotes peripheral T_{reg} generation via inhibition of histone deacetylases (HDAC) inhibition (97) or the induction of tolerogenic DCs (90) in mice. Similarly, *in vitro* studies show butyrate to be a potent inducer of human tolerogenic DCs by inducing RALDH activity and type 1 regulatory T cells (Tr1) differentiation via simultaneous HDAC inhibition and signaling through GPR109A (98). Previous gut microbiome analysis revealed a significant reduction in the number of butyrate-producing bacteria, such as *Firmicutes* and *Lachnospiraceae* in the colon of patients with IBD (24) and colorectal cancer (25). The gut microbiota through GPR109A signaling have anti-inflammatory properties by promoting colonic macrophages and DCs to induce differentiation of T_{reg} cells and immune-regulatory IL-10-producing T cells (23). GPR109A was also essential to induce IL-18 via a butyrate-mediated pathway in the colonic epithelium for intestinal integrity. Besides butyrate, GPR109A also acts as a receptor for niacin (or vitamin B3), which is also produced by the gut microbiota. Accordingly, niacin-deficient ($Niacr1^{-/-}$) mice displayed exacerbated DSS-induced colitis pathogenesis. Complete ablation of the gut microbiota or depletion of dietary fiber greatly increased the risk for DSS-induced colitis and colon cancer in mice (23). As previously discussed, there is a duality in the role of IELs in infection. We and others have found that a high acetate-yielding diet reduced colonic atrophy and inflammation in mice affected with *C. rodentium* infection in a GPR43-dependent manner (99) (K. H. McLeod, Y. Yap & E. Mariño, paper under review). SCFAs mediated by GPCRs conditioned the IECs to produce inflammatory cytokines and chemokines such as IL-6, CXCL1 and CXCL10 for a timely response against bacterial products in the protection against infection; thus highlighting again the critical connections between intestinal immunity, the gut microbiota and diet (99).

As previously mentioned, the SCFAs can exert their anti-inflammatory properties through HDAC inhibition (97, 100, 101). We have shown that feeding mice a high fiber diet enhanced T_{reg} numbers and function, which protected them against allergic airways disease (AAD) (89). In that study, we demonstrate that dietary acetate inhibited HDAC9 by increasing acetylation at the Foxp3 promoter, which led to increased T_{reg} cells (89). Further analyses demonstrated an increase in fecal and serum SCFA concentrations, particularly acetate, and identified the dominance of an acetate-producing bacteria, *Bacteroides acidifaciens* A40(T) strain in mice fed a high fiber diet. Likewise, another study reported that dietary fiber and the SCFA propionate protected against AAD in mice (102) in a GPR41-dependent manner. Dietary fiber decreased the ratio of *Firmicutes* to *Bacteroidetes*. Furthermore, mice treated with propionate had alterations in bone marrow hematopoiesis marked by enhanced generation of macrophage and dendritic cell (DC) precursors with impaired Th2 response in the lung. We observed in both mice and humans that a higher intake of dietary fiber during pregnancy was associated with a higher concentration of serum acetate and increased protection against the development of airway disease in offspring (89).

PUTTING THE BRAKES ON AUTOIMMUNE T1D

T1D is one of the most common autoimmune diseases that results in specific T cell-mediated destruction of pancreatic β-cells in genetically susceptible individuals (103). Although most of the action occurs in the pancreas and adjacent lymph nodes, the gut has been implicated as an early regulator of disease progression (104, 105). In fact, the progressive rise in global T1D incidence (106, 107) points to environmental factors, such as infections, antibiotics and diet, having a great impact on the development of T1D (94, 108–110). We believe that studying the synergistic roles of the intestinal barrier, the microbiota composition and the mucosal immune system offer potential intervention targets to treat T1D. Evidence shows that NOD mice (5, 8–10) and children diagnosed with T1D have a distinct and limited gut microbiota diversity (13), with an increased ratio of *Bacteroidetes* to *Firmicutes*, compared to healthy individuals (14). Furthermore, metagenomic data showed a significant reduction in abundance of butyrate producers, such as *Faecalibacterium*, and mucin degraders, such as *Prevotella* and *Akkermansia* (15, 16). This is in contrast with other SCFA producers such as *Bacteroides* and *Alistipes*, which were more abundant in the feces of diabetic individuals (15). As mentioned previously, butyrate is central for epithelial integrity, therefore an altered gut microbiota composition may contribute to a "leaky" gut and aberrant intestinal immune system, all contributing to the etiology of T1D.

In T1D, increased gut permeability contributes to intestinal inflammation, shown in both animal models (11) and human patients (17). Likewise, intestinal infection that promote a "leaky" gut also promoted accelerated T1D in mice by increased activation and proliferation of diabetogenic $CD8^+$ T cells in the pancreatic lymph nodes (PLNs) (111). The role of the gut in the loss of tolerance to self-antigens that leads to autoimmune T1D is poorly understood. However, an unbalanced host-microbe interaction and excess of food and microbial antigens may skew the intestinal immune system toward a perpetually pro-inflammatory state (12). Likewise, children with T1D show overall increased intestinal immune activation by high IL-4, IL-1α and IFN-γ protein expression in the small intestine LPL (18). The number of regulatory $Foxp3^+$ T_{regs} are reduced and/or inactive in biopsied small intestine tissues in T1D patients (19). Moreover, it has been reported that children newly diagnosed with T1D had reduced $Foxp3^+$ T_{regs} and increased IL-17-expressing $CD4^+$ and $CD8^+$ T cells in the blood, indicating the absence of immune suppression mechanisms (20). In contrast, adoptive transfer of splenic IL-17-producing $TCR\gamma\delta^+$ cells did not exacerbate diabetes in NOD mice, but instead had regulatory effects through TGF-β upregulation (87).

Inducing mucosal immune tolerance through aerosolized insulin has been considered a therapeutic approach to treat T1D in secondary prevention studies. Interestingly, transfer of purified splenic $CD8^+\gamma\delta$ T cells from diabetic NOD mice treated with insulin aerosol suppressed diabetes. Protection was associated with reduced $insulin^+_{B9-23}CD8^+$ T cells, increased IL-4 and IL-10 cytokines and reduced GAD^+CD4^+ T cell proliferation (85). This study magnifies the role of the gut mucosal immunity in the development of T1D. As such, an impaired thymus, the site for T cell selection and maturation including the IELs, accelerates autoimmune diabetes in mice. Remarkably, transfer of purified $TCR\gamma\delta^+CD8\alpha\alpha^+$ IELs from NOD mice prevented diabetes by reconstituting the small intestine IELs compartment of recipient mice that have undergone neonatal thymectomy (86). There is however, evidence to suggest a pathogenic role for $TCR\gamma\delta^+$ cells in T1D, as adoptive transfer of a specific subset ($CD27^-TCR\gamma\delta^+$) of these cells into NOD.SCID recipients accelerated T1D development. Besides, knockdown of the TCRδ receptor in NOD mice offered robust T1D protection (7). As the studies above have shown, there is more than meets the eye when it comes to the role of $TCR\gamma\delta^+$ cells in the story of T1D pathogenesis.

Our research group has focused on new insights studying the connectivity between the gut microbiota, mucosal immune system and diet for the prevention and cure of T1D. We were the first ones to elucidate that an altered gut microbiota and reduced production of SCFAs are signatures of T1D in NOD mice as it has been recently confirmed in individuals with T1D (21). We showed that exacerbated immune responses against islet antigens and increased gut leakiness were ameliorated by specifically augmenting the delivery of dietary acetate and butyrate. Therefore, we use a dietary intervention as an alternative therapeutic approach to halt immune-mediated beta-cell damage, promote a balanced and healthy gut microbiota, and abrogate the progression to clinical diabetes. We demonstrated that high SCFAs-yielding diets protected NOD mice from autoimmune diabetes (5). Acetate regulated autoreactive $CD8^+$ T cells through changes in B cell differentiation and function. On the other hand, butyrate enhanced splenic and colonic $Foxp3^+$ T_{reg} cell numbers through histone modification. Moreover, the diets high in acetate and butyrate significantly restored gut epithelial integrity by reducing circulating bacterial lipopolysaccharide (LPS) concentration, correlated an with increased expression of occludin. Recently we have shown, consistent with the previous results, that high butyrate-yielding diets increased the number of colonic $TCR\gamma\delta^+$ IELs associated with increased thymic IEL precursors (Y. Yap & E. Mariño, unpublished data). Overall, our results demonstrated the astonishingly beneficial effects of high SCFA-yielding diets, by targeting both the gut microbiota and the immune system, thus offering a promising outcome on the prevention and treatment of autoimmune and inflammatory diseases.

Whilst supplementation of dietary SCFAs proved to be a safe and non-invasive therapy in a mouse model of T1D (5), one cannot help but wonder if using dietary supplementation together with current pharmaceutical approaches will favorably and greatly increase treatment efficacy. Indeed, therapies combining dietary treatment with immunotherapy are now starting to be tested in various disease models. For example, a very recent study testing the combination of a ketogenic diet and PI3K inhibitors, such as metformin and SGLT2, demonstrated significantly enhanced efficacy/toxicity ratios in various murine models of cancer (112). If these findings were successfully, translation into human clinical trials may revolutionize our current therapeutic strategies to eventually pair dietary or medicinal food supplementation with targeted immunotherapy for the treatment of many autoimmune and inflammatory diseases.

CONCLUDING REMARKS

Altogether, these studies provide validation that the impact of dietary metabolites within the gut has local as well as far-reaching anti-inflammatory effects on host health. Taken together, these findings accentuate the powerful impact the triage of intestinal immune health, the gut microbiota and dietary factors have in the pathogenesis of various autoimmune and inflammatory diseases such as T1D, infections and IBD. Much effort has been made in recent years to uncover the exact mechanisms behind their effects on health and disease. We are advancing on sophisticated methods such as genomics, transcriptomics, proteomics, metabolomics and nutrition that allow us to understand how this dynamic trio works together. While more studies in humans are required, dietary SCFAs have shown to be promising novel therapeutics. Dietary SCFAs therapy may be specifically tailored and designed, alone or in combination with potential immunotherapies, to prevent or cure diseases, where the future of medicine lies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Implications of Probiotics on the Maternal-Neonatal Interface: Gut Microbiota, Immunomodulation, and Autoimmunity

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Probiotics are being investigated for the treatment of autoimmune disease by re-balancing dysbiosis induced changes in the immune system. Pregnancy is a health concern surrounding autoimmune disease, both for the mother and her child. Probiotics for maternity are emerging on the market and have gained significant momentum in the literature. Thus far, evidence supports that probiotics alter the structure of the normal microbiota and the microbiota changes significantly during pregnancy. The interaction between probiotics-induced changes and normal changes during pregnancy is poorly understood. Furthermore, there is emerging evidence that the maternal gut microbiota influences the microbiota of offspring, leading to questions on how maternal probiotics may influence the health of neonates. Underpinning the development and balance of the immune system, the microbiota, especially that of the gut, is significantly important, and dysbiosis is an agent of immune dysregulation and autoimmunity. However, few studies exist on the implications of maternal probiotics for the outcome of pregnancy in autoimmune disease. Is it helpful or harmful for mother with autoimmune disease to take probiotics, and would this be protective or pathogenic for her child? Controversy surrounds whether probiotics administered maternally or during infancy are healthful for allergic disease, and their use for autoimmunity is relatively unexplored. This review aims to discuss the use of maternal probiotics in health and autoimmune disease and to investigate their immunomodulatory properties.

Keywords: probiotics, gut microbiota, maternal, neonatal, autoimmunity

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INTRODUCTION

Probiotic based therapies are emerging in modern medicine and have turned the heads of multiple large pharmaceutical companies to treat diseases associated with gut dysbiosis including colorectal cancer, multiple sclerosis (MS), inflammatory bowel disease (IBD), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and obesity (1–4). Probiotics provide the potential to compensate for the pathological problems of gut dysbiosis and restore balance to the microbiota of the intestinal tract (5).

The definition of a "probiotic" was formed at The Lactic Acid Bacteria Industrial Platform in 1988 (6), acknowledged in joint consultation of the World Health Organization and Food and

Agriculture Organization (7), and reapproved in 2014 by a panel of scientist in the International Scientific Association for Probiotics and Prebiotics (8). It resounds throughout literature as an organism that, when delivered in an adequate dose, confers a health benefit beyond normally acquired nutrition (1, 2, 9). Since the discovery of probiotics, a whole slew of healthy gut-promoting microbe-associated products are being investigated that fall under the umbrella term "pharmabiotics." Besides probiotics, pharmabiotics include prebiotics, synbiotics, and metabolites (1). Another non-living pharmabiotic has recently been described as "paraprobiotic" or "ghost probiotic." Ghost probiotics are comprised of dead cells and bacterial metabolites and named for the interaction with living organisms of non-living material that was once a living microbe (10, 11).

This review does not focus on all of the aforementioned probiotic related products, but galvanizing research is being conducted on how metabolites from probiotics can confer protective effects upon their host. Recently, *Lactobacillus acidophilus* bacteriocin was proven to inhibit the growth of opportunistic pathogens *Gardnerella vaginalis*, *Streptococcus agalactiae*, and *Pseudomonas aeruginosa* which are also commensal (12). Metabolites isolated from cultured *Bacillus coagulans* drove maturation of monocytes (13). Short chain fatty acids, like butyrate, are another metabolic derivative of probiotics that are implicated in reducing disease severity in cancer and autoimmunity (14, 15). Butyrate has been shown to regulate the diversity of bacteria species in the gut as well as improve the function of the intestinal epithelial layer (16). Cell surface components and supernatant containing bacterial metabolites differentially stimulate immune cells by prompting either pro- or anti-inflammatory cytokine production (17). Therefore, metabolites perform a variety of beneficial functions including antimicrobial activity, enhancing the gut barrier, modulating microbiota diversity, and immunomodulation, all of which are implicated in the amelioration of autoimmune disorders. A detailed discussion on how bacterial metabolites may affect health and autoimmune diseases can be found elsewhere (14, 18).

Current clinical trials involving probiotics include using pet dogs to modulate the microbiota in a probiotic capacity in the elderly, prevention of necrotizing enterocolitis in preterm infants, and intervention for anxiety and depression (19–21). The only clinical trials investigating probiotics in autoimmunity are two studies on type 1 diabetes (22, 23), and one study on IgA Nephropathy (24). While the benefits of probiotics for intervention of gut dysbiosis are established, all angles of probiotic use need to be closely examined. Here we review probiotics influence on the gut microbiota, immune system, and autoimmunity in the normal, maternal, and fetal context.

PROBIOTICS

Changes to Gut Microbiota

Ten trillions of microbes inhabit the human gastrointestinal tract, the majority of which are bacteria (25). Difference in the gut microbiota occurs throughout the gastrointestinal tract. From the oral cavity to the colon, the microbiota changes. The esophagus, duodenum, and jejunum contain *Streptococcus* as the predominant genus, whereas the stomach is colonized by *Streptococcus* and *Prevotella* (25, 26). However, individuals who are carriers for the species *Helicobacter pylori* in their stomach have more bacteria from the phylum Proteobacteria (25, 26). The colon contains the richest diversity and highest abundance of bacteria, the bulk of which are from the phyla Firmicutes and Bacteroidetes (25, 26). Firmicutes is mostly comprised of gram positive species from several notable genera like *Enterococcus, Helicobacterium, Streptococcus*, *Staphylococcus, Lactobacillus*, *Clostridium*, *Lachnospireceae*, and *Streptococcus* (27). The phylum Bacteroidetes is comprised of gram negative rods, of which the most notable genera found in the gastrointestinal tract are *Bacteroides* and *Prevotella* (28). Differences in microbial populations also exist between the lumen and epithelium. A study using fluorescent *in situ* hybridization on sections of the colon showed that from the epithelium to the lumen the diversity of bacteria increased (26, 29). In the distal colon, a layer of interlaced bacteria barred the mucosa from *Bacteroides* and *Clostridium difficile* (26, 29). No bacterial probes hybridized with the mucosa itself, but bacteria present in the crypts—*Lactobacillus, Coriobacterium, Phascolarcotbacterium, Clostridium*, and assorted *Proteobacteria* genera—were also present in the interlaced layer and luminal content (26, 29). All of the bacteria in the interlaced layer, which included all those found in crypts as well as more species of *Clostridium* and some *Bacteroides*, were also found in the lumen (26, 29). The luminal content contained the highest number of different bacteria and included prominent genera such as *Bifidobacterium* and *Enterobacteriaceae* (26, 29). Other techniques of analyzing mucosa-associated microbes include washing away the luminal content and scraping the mucosa under sterile conditions or do not include the cross-sectional refinement of the aforementioned study (30, 31).

As an organ, the health of normal gut microbiota can be enhanced by probiotics. Probiotics have the capacity to antagonize harmful bacteria in the gut by secretion of antimicrobials, competitive adherence to the mucosa, strengthening of the epithelial layer, and modulation of the immune system (9). Probiotics have been shown to stabilize bacterial populations during changes in diet (32) and at the same time change the architecture of the gut microbiota (33). Probiotic mixtures of *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* species alter the landscape of the gut microbiota by increasing the number of Firmicutes and Actinobacteria while decreasing the number of Bacteroidetes and Proteobacteria (33–35). This same shift in bacterial populations is seen both in healthy controls as well as diseased individuals (36). Probiotics increase the number of *Lactobacilli* and *Bifidobacteria* secreted in feces, indicating their capacity for colonization of the human gut, which can be enhanced by coating the bacteria in a capsule that protects them from the gastroduodenal environment (37). In colorectal cancer patients, treatment with *Lactobacillus acidophilus*, *Bifidobacterium longum*, and *Enterococcus faecalis* decreased the abundance of pathogenic *Fusobacterium*, the prevalence of which is associated with both IBD and colorectal cancer (2). Besides influencing bacterial

populations of the gut, probiotics may also ameliorate disease-associated imbalances in the fungal microbiota. A recently published study showed a probiotic mixture of *Lactobacillus rhamnosus* and *Bifidobacterium animalis* decreased serum IgG levels against *Candida albicans* in schizophrenia associated dysbiotic conditions (38).

Most studies with probiotics involve using them either on patients or animal models of disease. The challenge is to delineate how different species and genera impact the normal gut microbiota. For example, treatment of obese mice on a high fat diet with two strains of *Lactobacillus*, *L. curvatus* and *L. plantarum*, changed the microbiota by increasing *Lactobacillus* species, Clostridiales including Ruminococcaceae and Lachnospiraceae, and *Bifidobacterium* (39). Other studies in mice found that treatment with *L. acidophilus* may have decreased the abundance of *Lactobacillus* (40). Increases of Lachnospiraceae with *Lactobacillus* treatment was supported in a study on *L. rhamnosus* GG (LGG) treatment of patients with cirrhosis that found increases in Lachnospiraceae and Incertae sedis Clostridia XIV, while the abundance of Enterobacteriaceae and Porphyromonadaceae decreased (41). This study did not describe any changes to Lactobacillaceae. Intervention with *Bifidobacterium bifidum* at the phylum level increased the ratio of Firmicutes to Bacteroidetes, and at the genus level decreased *Bacteroides, Faecalibacterium*, and *Lachnospira* with increasing *Ruminococcus*, *Dorea*, and *Streptococcus* (42). Interesting, directly post intervention there was a transient rise in *Bacteroides* and *Lachnospira* before the abundance decreased. Thus, while it is difficult to account for difference in dose and disease, probiotic *Lactobacillus* and *Bifidobacterium* differentially alter the gut microbiota leading to the conclusion that selection of probiotics must consider the pathology of dysbiosis.

Modulation of Immune System

Most probiotic strains of bacteria are gram positive bacteria, meaning that they will interact with Toll-like receptor (TLR)-2, responsible for recognition of bacterial peptidoglycan, and produce pro-inflammatory cytokines, such as IL-6 (43, 44). Several studies have shown that TLR2 is upregulated in macrophages and epithelial cells after treatment with probiotic species (45–47). Paradoxically, TLR2 stimulation is known to activate NF-kB through a tyrosine kinase dependent manner (48), but NF-kB signaling, along with resulting pro-inflammatory cytokine expression, has been shown to be downregulated with probiotic treatment with these bacteria (49, 50). Ryu et al. showed that, in mice, pathological consequences of *C. rodentium* infection is decreased by pretreatment with LGG. Although the study showed that histological and mortality was not improved in TLR2 knockout (KO) or TLR4 KO, pro-inflammatory TNF-α, IFN-γ, and MCP-1 were downregulated in the TLR4 KO, but not the TLR2 KO (51). Therefore, down regulation of pro-inflammatory cytokine production resultant from activation of NF-kB may be dependent on TLR2 stimulation. Actually, probiotic treatment upregulates anti-inflammatory molecules such as IL-10 and TGF-β and downregulates pro-inflammatory molecules such as IL-8, IL-1β, and TNF-α (46, 52). Yet, probiotics do not completely truncate pro-inflammatory signaling as upregulation of IFN-γ and IL-2 was observed in the Peyer's patches of mice treated with *L. casei* supplemented yogurt (53), and they have been shown to enhance phagocytic activity of granulocytes and monocytes (54). Taken together, these studies suggest that the anti-inflammatory effects of probiotics may be due the development of immune tolerance by a refined modulation of pattern recognition receptor (PRR) activation or by another mechanism altogether.

Probiotics also modulate the adaptive immune system by increasing the ratio of regulatory to effector T cell populations (52, 55–58). One putative mechanism of increasing T regulatory (Treg) cells is through inducing regulatory dendritic cells (DCs), which were determined via co-culture by Kwon et al. to cause differentiation of T cells into inducible Treg (iTreg) cells in peripheral tissue by upregulating forkhead box P3 (Foxp3) (52, 56, 59). The upregulation of Treg cells occurs concomitant with suppression of effectors cells such as T-helper (Th)1, Th2, and Th17 (60). Imbalances in these populations associated with disease are restored by probiotics (55, 61). Furthermore, some probiotics beneficially enhance B cell responses. Asthma patients treated with *Clostridium butyricum* enhanced immunotherapy by improving serum-specific IgE, increasing IL-10 producing B cells, and enhancing antigen specificity in peripheral blood B cells (62). A strain of heat killed *L. paracasei* isolated from a human adult induced increases in antigen-specific IgA production in mice as well as increases in T follicular helper (Tfh) cells (63). *L. plantarum* strain AYA was similarly found to increase differentiation of IgA producing B cells into plasma cells resulting in increased IgA production in the small intestine and lung, thus providing protection against infections (64). Altogether, probiotics induce regulation of the immune system by promoting shifts in lymphocyte populations and promotion of an anti-inflammatory environment.

Effect on Autoimmunity

Autoimmune disorders are believed to result from genetic predisposition and interactions with the environment (65, 66). Dysregulation of commensal microbial communities and interactions with pathogens are an emerging hallmark of autoimmune disease (67). Many autoimmune disorders have recently been associated with gut dysbiosis including SLE, type 1 diabetes, RA, and MS (68–71). Some autoimmune disorders are thought to be catalyzed through molecular mimicry by microbes. For example, 50–70% of cases of Guillain-Barré syndrome, an autoimmune condition and complication of Zika virus infection, occur after either a respiratory or gastrointestinal infection (72). Instances of Guillain-Barré have been associated with Zika virus, *Campylobacter jejuni*, cytomegalovirus, Epstein-Barr virus, measles, influenza A, and *Mycoplasma pneumoniae* (72). Acute rheumatic fever, another autoimmune driven illness, occurs after *Streptococcus pyogenes* infection (66). Arthritis has also been associated with infection of gut pathogens such as *Shigella*, *Salmonella*, *Yersinia,* and *Campylobacter* and synovial fluid has been found to contain antigens from these pathogens (73, 74).

Disrupting autoimmune disorders through probiotic intervention has generated a lot of interest as a healthcare strategy for chronic autoimmune disorders such as SLE (75).

Our lab has shown that *Lactobacillus* species can beneficially impact the progression of glomerulonephritis, an inflammatory kidney syndrome occurring in over half of all SLE patients, in MRL/lpr mice (61). Other labs have shown the beneficial effects of probiotics on SLE in NZB/W F1 mice, another classical SLE mouse model (76, 77). Cardiac cell apoptosis in NZB/W F1 mice was attenuated by *L. paracasei* (76) and hepatic cell apoptosis was ameliorated by mixture of *L. paracasei* and two different strains of *L. reuteri* (77). Several studies have shown that probiotics may have a protective effect against respiratory infections (78, 79), which may mitigate the potential for those autoimmune disorders caused by molecular mimicry. At the frontier of probiotic research, ghost probiotics are being investigated for their potential to protect from infections associated with autoimmune disorders (10, 11).

One role of probiotics in dampening autoimmunity is through enhancing gut barrier function. Leaky gut, a condition characterized by a disrupted epithelial layer, is caused by loosening of the tight junction proteins that hold a contiguous barrier in the gastrointestinal tract, and results in penetration of foreign antigens and harmful substances (80). This condition can arise from diet and stress induced dysbiotic conditions and excessive inflammation (81, 82). In autoimmune disease, a leaky gut can be either etiological or aggravating (80), and is characteristic in mouse models and humans with autoimmune disease (61, 83). Pathobionts like *E. gallinarum* translocate into non-gut tissues under leaky gut conditions and exacerbate disease in a lupus mouse model (NZWxBXSB) (83). We have previously shown that *Lactobacillus* species can effectively improve the intestinal barrier of a different strain of lupus-prone mice, MRL/lpr (61). The capacity for probiotics, prebiotics, and metabolites to enhance gut epithelial barrier function is an indication for their effectiveness in treating autoimmune diseases (61, 70).

Probiotics have been shown to be beneficial in a number of autoimmune diseases. In SLE, the ratio of Th17 to Treg cells is increased (84), but can be restored by probiotic mixture of *Lactobacillus* species (61). In a clinical trial, treatment with *L. casei* significantly decreased TNF-α and IL-10 in patients with RA (85). Although IL-12 significantly decreased in both the control and probiotic group, the ratio of IL-10 to IL-12 at the end of the study was significantly higher in the probiotic group (85). The clinical course of MS is improved by shifting a Th1 response to Th2 and, among drugs such as glatiramer acetate, probiotics also have the potential for this particular immunomodulation (86). For example, a phase I clinical trial using probiotic helminths has shown increases in IL-4 and IL-10, Th2 signature cytokines (87). Experimental models of MS also showed promising immunomodulatory effects of a mixture of probiotics called IRT5 (*L. casei, L. acidophilus, L. reuteri, B. bifidum, Streptococcus thermophiles*) by balancing Treg to the effector Th1 and Th17 cells (88). Early probiotics have also been associated with protection against type 1 diabetes islet autoimmunity in an international longitudinal observational study on children at risk (89). Based on these evidences, probiotics may serve as a means of correcting the effects of autoimmune-related dysbiosis and promoting the health of patients suffering from autoimmune disorders or diseases. However, there is little research investigating the impact of maternally administered probiotics on the development of autoimmunity in mothers and infants.

MATERNAL PROBIOTICS

Pregnancy Induces Changes in Gut Microbiota

The normal microbiota undergoes changes during pregnancy including increases in oral presence of *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans,* and *Candida*; anaerobic and aerobic bacteria increase in the placenta; the gastrointestinal tract increases in Actinobacteria, Proteobacteria, and decreases in *Faecalibacterium*; and the vaginal microbiota increases in *Lactobacillus* species that gradually decline in presence postpartum (90, 91). Beta-diversity, the differences in microbial composition between samples, increases in the third trimester compared to the first trimester (90, 92). *Streptococcus, Lactobacillus,* and *Enterococcus* are enriched during the third trimester, and *Streptococcus* abundance is still enhanced 1 month postpartum, but the abundance of *Faecalibacterium*, which produces butyrate and promotes an anti-inflammatory environment, is reduced (90). Proteobacteria are associated with increased inflammation and transferring the human gut microbiota of the first and third trimester to germ-free mice induced a higher inflammatory response (increases in IFN-γ, IL-2, IL-6, and TNF-α) in mice receiving the third trimester gut microbiota (90). Studies in German Shepherd dogs revealed that bacterial communities shift after giving birth; Fusobacteriae and Bacteroidetes decreased while Firmicutes, especially Lactobacillaceae increased (93). It has been suggested that gut microbes have the potential to colonize the vagina or spread by translocation across the epithelium into the blood stream, and this dissemination has the potential to impact pregnancy outcomes (94).

Several probiotics for maternity have appeared on the market while the research on the effect of probiotics on maternity and infants remains slim. How maternal probiotics modulate changes to the gut microbiota that occur during pregnancy must be studied. Probiotics do not, however, significantly alter the vaginal microbiota during pregnancy (95). Neither do they have a significant impact on glycemia or health of offspring in obese pregnancy (96), however a more comprehensive study including a longer probiotic administration period promises to be more revealing (97). Also, the addition of dietary counseling to probiotic consumption of *Lactobacillus rhamnosus* and *Bifidobacterium lactis* in pregnant women with metabolic disorder decreased fasting blood glucose, improved glucose tolerance, and decreased the frequency of gestational diabetes mellitus (98, 99). Although probiotics have been proposed to enhance the integrity of the gut epithelium barrier, studies in overweight pregnant women revealed that probiotics consisting of *Bifidobacterium animals ssp lactis* and *Lactobacillus rhamnosus* failed to reduce the increases in levels of serum zonulin and lipopolysaccharide (LPS) associated with pregnancy (100),

both of which suggest impaired intestinal barrier function. Furthermore, infection is an important factor in pre-term labor and pathogenic microbes can be outcompeted by probiotics, yet, according to a systematic review by Jarde et al. (101) probiotics neither increased nor decreased pre-term birth (101). Kriss et al. (102) corroborates this conclusion, but suggests that high probiotic yogurt consumption may be associated with reduction in preterm delivery in Mexican women of normal weight as opposed to overweight (102). Altogether, limited evidence exists that maternal probiotics beneficially impact the mother-infant interface and more research is needed on how changes in the gut microbiota that occur during pregnancy might be modulated by probiotic treatment.

The Immune System During Pregnancy

The immune system undergoes hormone related changes during pregnancy to support and tolerate the developing fetus. Prior to our current understanding, it was believed that the mother's immune system was inactivated (103). In the 1980's it was believed that the overwhelming immune environment of the uterus was anti-inflammatory, or resembling a Th2 (high IL-4 and IL-10) response. However, current research testifies to a coordinated pro- and anti-inflammatory response. Characteristic phases mark the progression of pregnancy, starting with an inflammatory phase during implantation with high levels of the cytokines IL-6, IL-8, and TNF-α (103, 104). This pro-inflammatory state is critical for the blastocyst to rupture the uterus epithelial layer and for subsequent tissue repair (103, 104). The second phase is predominantly anti-inflammatory with markedly higher immune tolerance to facilitate rapid growth of the fetus (103, 104). In the third and final stage, pro-inflammatory immune responses are part of promoting contraction and rejection of the placenta (103, 104).

The composition of the uterine immune system during normal pregnancy is dominated by uterine natural killer (uNK) cells which aid in trophoblast invasion of the uterus (104–106) and are tolerogenic in the uterus and pro-fetus, supposedly due to a high presence of IL-10 (106). However, when these cells are relocated to non-fetal environment they resume their cytotoxic function (106). uNK cells expresses the surface marker phenotype of cytokine-producing NK cells ($CD56^{high}CD16^{-}$), but are similar to cytotoxic NK cells ($CD56^{low/-}CD16^{+}$) and differ from cytokine-producing NK cells circulating in the blood due to the presence of granules (107). In 2005, NK and NKT cells were proposed to fall into different subsets based on Th1- and Th2- like cytokine profiles (108). The NK1/NKT1 subtype produces IFN-γ and the NK2/NKT2 subtype produces IL-5 and IL-13. Furthermore, studies differentiating these subtypes showed decreases in the ratio between NK1/NKT1 and NK2/NKT2 cells were associated with healthy pregnancy (109).

Macrophages are the most abundant antigen presenting cell (APC) type, and, next to NK cells, they are the second most abundant leukocytes (105). Uterine macrophages are similar to M2 macrophages, meaning their phenotype is selective for tissue repair and is highly associated with a classical Th2 response and, like uterine DCs (uDCs), express IL-10, TGF-β, and indolamine 2,3-dioxygenase (IDO) (106). However, unlike M2 macrophages, uterine macrophages are not induced by Th2 cytokines but by macrophage colony-stimulating factor (M-CSF) and IL-10 (107). Uterine macrophages, uNK, and uDCs all play a key role in decidual formation (104). Uterine macrophages and uNK cells also facilitate remodeling of vasculature, especially spiral arteries, transient arteries that supply blood to the endometrium and decidua, through expression of molecular compounds important for angiogenesis (107, 110).

In regard to the adaptive immune system, $CD8^{+}$ T cells are more abundant in the pregnant uterus than $CD4^{+}$ T cells (105). However, the populations of helper $CD4^{+}$ T cells undergo dynamic modulation during pregnancy (105). For one, a Th2-like immune profile dominates over Th1, and, in fact, over-expression of the cytokines associated with Th1, IFN-γ and IL-2, leads to spontaneous abortion (105). Levels of Th17 cells rise in the decidua and peripheral blood during the first trimester, and, by the third trimester, fall back to normal levels (111). While an unbalanced Th17 response is associated with pathological conditions including autoimmune diseases, increases in Th17 cells during early pregnancy may be necessary for the events that take place at this time, like the invasion of trophoblasts (105, 111, 112). $CD4^{+}CD25^{+}$ Treg cells also increase during the first trimester, but unlike their Th17 cousins, Treg levels peak during the second trimester in the decidua and peripheral blood (105, 111, 112). Treg cells stimulate uDCs to express IDO, which inhibits T cell proliferation and is also expressed in trophoblasts and macrophages (113). The mechanism of IDO activity is by catalyzing the conversion of tryptophan to kynurenine, an agonist for aryl hydrocarbon receptor (AhR) (114, 115). AhR has been reported to have immunosuppressive and immune-modulatory activity through the induction of TGF-β, IL-10, and IL-22 (114, 116).

Our knowledge is limited on how maternally administered probiotics interact in an immunomodulatory way with pregnancy-associated changes to the immune system. However, the probiotic bacterium *Lactobacillus reuteri* can catabolize tryptophan to indole-3-aldehyde (IAld) under high tryptophan conditions when sugars are depleted as an energy source, and, like kynurenine, IAld is another agonist of AhR (114, 115). Probiotics may therefore be able to enhance the immunosuppressive environment of the uterus either through direct colonization of the placenta and amniotic fluid, or by a peripheral interaction. The mechanisms by which bacteria affect the prenatal environment are further discussed in following sections. Of the few clinical studies in this area, Rautava et al. (117) found that expression of TLR1, TLR7, and MD-2, a surface protein that associates with TLR4 to confer a signaling response to LPS, were decreased in the placenta of mothers receiving *B. lactis*, but TLR3 expression increased. A combination of *B. lactis* and LGG decreased the transcript levels of TLR1, MD-2, and TIR domain-containing adaptor protein (TIRAP), an adaptor protein that connects TLR4 and TLR2 with MyD88 (117). Furthermore, post-delivery treatment with fermented milk with *L. casei* DN11201 increased NK cell levels in the blood and, only at 10 days postpartum, increased IgG4 levels, but the treatment failed to significantly alter the numbers of T and B lymphocytes

as well as the ratio of Th1 to Th2 cells and related cytokine levels (118). At 45 days postpartum, breastmilk had significantly increased levels of IL-10 and TNF-α in the group receiving fermented milk, but levels of TGF-β, IL-1, IL-6, IL-8, IL-12, and IgA were unchanged (118). However, VSL#3—a mixture of *Lactobacillus*, *Bifidobacterium*, and one *Streptococcus* species significantly reduced the decrease of Th2-like cytokines, IL-4 and IL-10 (95). In summary, the tolerogenic properties of probiotics may enhance the tolerogenic maternal state, but it is unclear whether they inhibit the necessary pro-inflammatory stages of pregnancy.

Pregnancy and Autoimmunity

Pregnancy is a contentious topic when it comes to autoimmune disease, especially since many autoimmune diseases, include SLE, RA, and systemic scleroderma, are female-biased (119, 120). These three aforementioned autoimmune diseases are particularly involved with pathogenesis of various kinds of kidney disease, and the kidney is a particularly vulnerable organ during the stress of pregnancy (119). The current recommendation for women with autoimmune disease is to avoid pregnancy, especially in the case of kidney disease, because it may lead to infertility and complications for the pregnant woman and her child (121–126). However, recent evidence shows that serum creatinine and glomerular filtration rate were significantly improved in pregnant patients with lupus nephritis compared to non-pregnant lupus nephritis patients (127). Pregnancy also failed to significantly exacerbate renal flares compared to control patients in lupus nephritis patients (127). Furthermore, pregnancy has been found to dampen symptoms of autoimmune diseases, such as SLE and MS, that are pathologically dependent on imbalanced Th1 and Th17 responses (113). Thus, there is conflicting evidence on how pregnancy impacts autoimmune disease and the current recommendation for women with autoimmune disease is to avoid pregnancy.

While we know that both pregnancy and probiotics may dampen autoimmune disease, the interaction between probiotics and pregnancy in autoimmune disease is poorly understood. It is therefore critical that we seek to understand how the autoimmune ameliorative effects of probiotics impact pregnancies associated with autoimmune disorders.

EFFECT OF MATERNAL PROBIOTICS ON OFFSPRING

Neonatal Development of the Gut Microbiota

The fetus, formerly believed to be sterile, was found to be colonized by bacteria via the placenta and amniotic fluid through groundbreaking research conducted in the early 2010's (128, 129). Up until this point, it was believed that infants' microbiota began with bacteria from their mothers' vaginal canal (130). The discovery that smashed the sterile womb hypothesis was followed by several ideas on how early colonization of the fetal gastrointestinal tract may occur including swallowing amniotic fluid (131), or translocation from a pregnant mothers' gastrointestinal tract to the mesenteric lymph nodes (MLN) and to the mammary glands by phagocytic immune cells (132). Similarities in bacterial load were found between infant feces and the feces, milk, and blood of the mom (132). Supporting this hypothesis, the bacterial load of MLNs in mice was determined to be higher during late pregnancy followed by a steep decline after birth, concomitant with a sharp increase of bacteria present in mammary tissue during lactation (132). Additionally, hormonal changes may increase permeability of the gut epithelium during pregnancy and lactation, further facilitating transfer of bacteria from the gut to the mammary tissue (133). Two studies support a transfer system from the gut to the mammary gland: one showed genetically labeled *Enterococcus faecium* in the meconium of pups born to mice orally gavaged with the bacteria (134), and the other, a more recent study, traced the tissues that fluorescently labeled *Lactobacillus lactis* and *L. salivarius* translocated to when administered via gavage to pregnant BALB/c mice (135). Are these translocated bacteria artifacts of active phagocytic cells or is there an alternative mechanism of transport? While this question remains unanswered, some evidence contradicts the hypothesis of translocation from the gut through the MLN to the mammary glands. For example, Treven et al. (35) conducted a study in which pregnant mice gavaged with 4×10^8 colony forming units (cfu) per dose of either LGG or *Lactobacillus gasseri* K7 (LK7) were sacrificed at 3 and 8 days postpartum. Bacteria from the MLN and mammary gland were assessed. Neither LGG nor LK7 were found in the probiotic treated MLN or mammary gland, yet bacteria from the genus *Lactobacilliceae* were only present in the mammary gland of probiotic treated mice (35). Similarly, treatment of women with VLS#3 during pregnancy showed that the probiotic organisms administered did not enter the mammary gland, but the probiotic group had higher numbers of *Lactobacilli* and *Bifidobacteria* detected in the colostrum of milk (136). However, this contradiction may be explained if translocation is a transient phenomenon, as Perez reported his findings from 1 day postpartum and Treven et al. (35) reported his findings from 3 to 8 days postpartum. In summary, there is evidence that infants receive their microbiota, both through prenatal exposure and through nursing, from the bacteria that colonize the mother's gastrointestinal tract (**Figure 1**). However, the evidence is not conclusive. As previously mentioned, probiotics were also found to alter the vaginal microbiota, and, therefore, influence the development of the infant's gut microbiota.

With this in mind, literature also supports that the gut microbiota of vaginally delivered newborns are closer in resemblance to the mother's vaginal microbiota than her gut, and newborns delivered by Cesarean section have a microbiota most similar to the skin microbiota (130, 137). In general, the microbiota of an infant lacks the diversity of an adult (93), and the infant's microbiota is most similar to their mother's microbiota at 4 years old (90). It does not reach full diversity until after 4 years of age (138). One study showed the probiotic *L. casei subsp. rhamnosus* administered to infants from birth to 6 months greatly altered the gut microbiota, significantly increasing 682 taxa including other lactic acid bacterial species

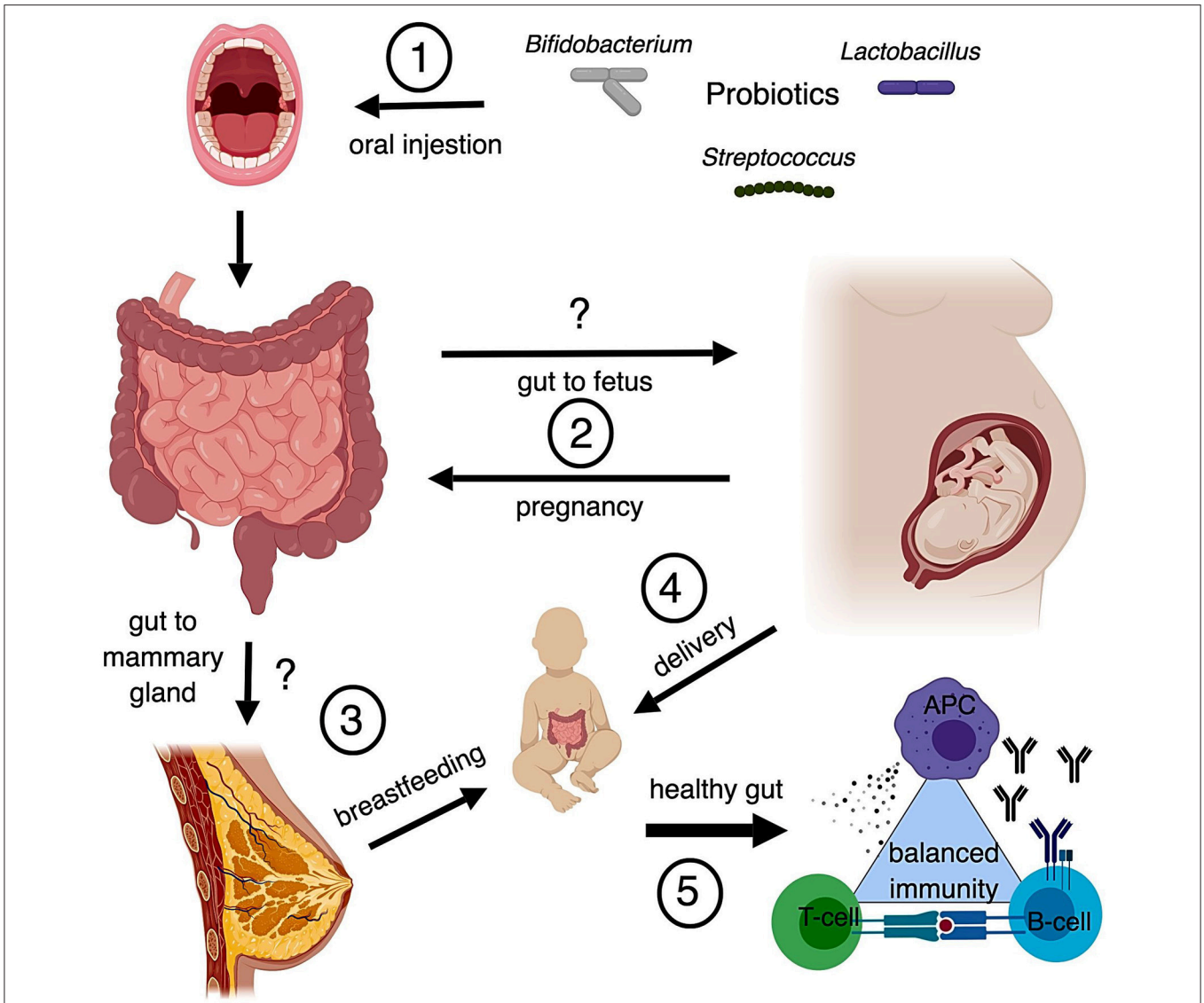

FIGURE 1 | Possible routes of maternal probiotics to neonate (1) Common probiotics include bacteria from the genera *Bifidobacterium*, *Streptococcus*, and *Lactobacillus*. Oral probiotics modulate populations of bacteria in the gut. (2) Translocation of bacteria from the gut to the fetal environment is controversial, however there is also the possibility that metabolites derived from gut bacteria can influence fetal development as well. While there are known changes to the gut microbiota that occur with pregnancy, the modulation of gut microbiota by probiotics during pregnancy is understudied. (3) Translocation of bacteria from the gut to the mammary gland is also controversial. Studies suggest similarities in the microbial composition between the two and that breastfeeding provides important immunological protection through maternal antibodies and microbes. (4) The method of delivery impacts the development of the neonatal gut microbiome. Infants receive microbiota from their mother's vagina unless they are delivered by C section. Changes to vaginal microbiota from probiotic treatment is controversial. (5) Development of the infant's gut microbiota is critical to forming and maintaining a balanced immune system. Disruption of gut microbiota early in life, through C section or antibiotics, may be linked to autoimmunity and hyperallergenic responses.

(139). Proteobacteria represented 60% of the taxa that were significantly increased (139). Another study on the long-term effects of probiotics on atopic children showed that probiotics did not significantly alter the diversity of bacterial phyla and that the gut microbiota is most unstable during the first two years of life with rather dramatic fluctuation in major phyla (140). Also, there was no long-term effect of probiotic treatment on the composition of the gut microbiota (140). However, probiotics did result in the removal of *Lactococcus* from the probiotic treated group during intervention (140). Another study showed similar results, the composition of the gut microbiota changed dramatically over time but retained a notable number of bacterial species from birth to 3 years of age (141). Also, the functional metabolic pathways remained static even though the bacterial communities changed (141). Other studies, such as a placebo-controlled study in Finland and Germany, found that treating mothers with probiotics during lactation significantly altered populations of bacteria, specifically *L. rhamnosus* and *B. longum*

given to Finnish women was reflected in the offspring by higher percentages of *Lactobacillus* and *Enterococcus* and lower levels of *Bifidobacterium* (142). Interestingly, the effects varied between the two countries due to differences in the way that infants were fed and seeding microbiota (142). In mice, the colonic gut microbiota was examined with treatment of probiotic fermented milk with *L. casei* DN-114001 (143). This study included four groups: no treatment, only pups received probiotics, only dams received probiotics, and both dams and pups received probiotics (143). *Bifidobacteria* and *Enterobacteria* levels were significantly increased in both groups where probiotics were fed to dams (143). After weaning, groups that did not feed probiotics to the pups saw a decline in *Bifidobacteria* and *Enterobacteria* levels (143). Similarly, maternal treatment with *Clostridium butyricum* increased levels of *Lactobacillus* and *Bifidobacterium* in pups while decreasing levels of *Enterobacter* spp (144). However, after weaning, the group giving *C. butyricum* only to pups overtook all other groups in the levels of *Bifidobacterium* and *Lactobacillus* (144). In all, the infant gut microbiota may be more susceptible to changes in structure, but not function, than the adult gut, and therefore easily influenced by probiotic intervention.

The health implications for either maternally administered or early childhood probiotics are diverse. *L. rhamnosus* when administered at 10^{10} cfu every day for 4 weeks before birth and then continued for 6 months either to breastfeeding mothers or in water given to offspring moderated excessive weight gain in offspring (145). Furthermore, one of the most significant advances in infant probiotic therapy is the mounting evidence that they can reduce the incidence of necrotizing enterocolitis in pre-term infants (131, 146). Besides development of a robust immune system, the infant gut microbiota is critical for angiogenesis in the gastrointestinal system and pre-term infants have a severely deficient microbiota infants (131, 146). Necrotizing enterocolitis is a condition in which the vasculature of the gastrointestinal tract is not well developed, as occurs in pre-term infants, and results in the death of gut tissue infants (131, 146). Studies in germ-free mice show that development of vasculature can be induced by colonizing the gut with normal microbiota, and that the gut epithelium is an important mediator between the microbiota and vasculature (147). Thus, the use of early probiotic intervention has made significant advances in developmental healthcare strategies, with the caveat that perinatal probiotic exposure can alter off-target developmental processes (148).

Modulation of the Developing Immune System

At birth, the immune system is naïve and the adaptive immune system is undeveloped. Specific differences exist in a neonatal innate immune system compared to an adult (149). Similar to the uterine environment, newborns have a higher number of NK cells than adults, and the NK cells are deficient in cytotoxic capacities, much like uNK cells (149). Neonatal blood contains more monocytes but less plasmacytoid DCs (pDCs) than adults (150). Additionally, neonatal whole blood monocytes, conventional DCs (cDCs), and pDCs are less responsive and less polyfunctional than their adult counterparts. Interestingly, these differences are most profound following TLR1/2, TLR4, and TLR7/8 stimulation and, for the monocytes and cDCs, only observed in umbilical cord whole blood—a proxy for neonatal blood—instead of PBMC isolated cells (150). This indicates a role for soluble factors that suppressed the responsiveness (151). In general, neonatal immune cells produced less type 1 cytokines, more Th17-inducing cytokines, and more IL-10 (150). As for the adaptive immune system, like the uterine environment, Th1 cytokines such as IFN-γ are reduced (152). Studies in rats showed that germinal centers in the spleen, lymph nodes, and Peyer's patches didn't develop until after 20 days of age, which is between the times when rats are weaned and reach sexual maturity (153). Interestingly, nascent follicles begin to form around seven days of age, which is about 5 days before rats begin to nibble on solid food (153). The beginning of eating a non-maternally supplemented diet marks the maturation of the immune system adaptive compartments and the end of provisional protection from a mother's milk. Prior to maturation, maternal antibodies are transferred to infants through milk and constitute early adaptive immunity (154). Specifically, IgA is the main antibody produced by the mucosal associated lymphoid tissue (MALT) of the mammary glands (154). IgA crosses the epithelium by binding to polymeric immunoglobulin receptor (pIgR) on the basal side of epithelial cells and transcytosis into the milk ducts (154). Early endogenous mucosal IgA production can be stimulated earlier in the absence of maternally transferred antibodies, such as the case when immunocompetent mice nurse on immunocompromised mothers (155). In this scenario, it is still unknown whether or not there is an immune stimulatory factor in milk that enhances early endogenous IgA production. However, it is known that the maternally transferred microbiota are responsible for jumpstarting the immune system (156). Translocation of bacteria from the gut to the MLN, where antigens from the bacteria are presented to educate adaptive immune cells, occurs spontaneously in infants during the nursing period (157). Therefore, the gut microbiota is important for education and maturation of the immune system.

Perinatal probiotics can shape the development of the immune system. Maternally administered *B. lactis* reduced TLR7 mRNA expression in the fetal intestines, whereas a combination of LGG and *B. lactis* decreased TLR6 mRNA expression (117). Postnatal probiotic supplemented food given to lactating mice significantly increased their offspring's Treg cell populations in saline control and peanut extract induced allergic response groups, but did not change inducible Treg cell populations (158). Probiotic treatment also decreased IL-6, IL-9, TNF-α, IL-17, and IL-7 mRNA expression in the pups' spleens (158). When probiotic fermented milk containing *L. casei* DN-114001 was administered to dams, the offspring had in increased level of secretory IgA in intestinal luminal fluid at 12 days of age, however at 28 days of age the same pups had a lower number of cells producing IgA in the small intestines (143). Additionally, when dams were fed probiotics their offspring had decreased numbers of macrophages at 12 days of age, but when probiotics were administered to postnatally the number of mature F4/80$^+$ macrophages was significantly increased by 45 days of age (143).

Contrary to these findings, *C. butyricum* treatment in either dams or pups, although it similarly increased levels of *Bifidobacterium*, did not significantly affect the levels of secretory IgA in intestinal fluid at 14 and 28 days of age (144). Probiotic milk containing a combination of 5×10^{10} cfu *L. rhamnosus,* 5×10^{10} cfu *B. animalis,* and 5×10^{8} cfu *L. acidophilus* per dose administered daily during pregnancy conferred a decreased risk of atopic dermatitis in infants and a decrease in the proportion Th22 cells, however no difference in other T helper cell subsets or Th1 to Th2 ratio was observed (159). *B. pseudocatenulatum* administered 2 days postnatally to C57BL/6 mice until 21 days old decreased stress hormones in a model for gut-brain involvement in early life trauma and chronic stress induced by maternal separation (160). The probiotic treatment also dampened LPS induced IFN-γ expression in MLN cells, which attributed to increased sensitivity to glucocorticoids (160). Altogether, probiotics received perinatally encourage endogenous adaptive immune response, enhance immune system maturation, and reduce pro-inflammatory immune responses. Yet, these results are still controversial and many discrepancies exist.

Influence of Maternal Probiotics on Autoimmunity in Offspring

While maternal probiotics have attracted attention for the health of newborns by reducing allergies and incidence of pre-eclampsia (161, 162), only one study has been has been reported on perinatal probiotics for autoimmune disease. This study found no association between incidence of celiac disease in children and maternally administered probiotics during late pregnancy and nursing (163). Although there is scant evidence for maternal probiotic intervention for autoimmune disease in infants, there is evidence that early microbial associated disruptions may be a contributing factor to the development of autoimmune disease. In children who develop type 1 diabetes, a decrease in the diversity of the gut microbiota occurs after seroconversion and before diagnosis (141). The bacterial genera *Blautia* and *Ruminococcus* were found in higher abundance in children who developed type 1 diabetes and contributed to increased serum levels of branched-chain amino acids (141). Furthermore, the genetic expression of the microbiota changes before the onset of clinical symptoms, and these changes include increases in sugar transport systems (141). Also, children born by C-section or exposed to prenatal antibiotics had increased risk of celiac disease, asthma and type 1 diabetes (164). Thus, evidence supports the importance of maternal transfer of the gut microbiota in prevention of autoimmune disease and creates a gap that could potentially be filled by probiotics intervention.

CONCLUSIONS

Significant grounds have been covered in establishing the link between dysbiosis and autoimmune disease marked by the presence of a leaky gut and systemic inflammation. From what we can study of the complex ecosystem that is the gut microbiota, probiotics induce significant changes to the normal microbiota and enhance anti-inflammatory immune reactions. Through their immunological modulatory capacity, probiotics can therefore alleviate autoimmune disease severity.

The normal gut microenvironment undergoes specific changes during maternity including an increase in gut barrier permeability. Furthermore, specific changes in the immune system occur to support and tolerate the developing fetus. Maternally administered probiotics may impact the health of a child by altering the bacteria that initially colonize the developing infant. Maternal probiotics thus have the potential to influence neonatal immune development since the initial programming of the immune system is dependent on the gut microbiota.

As the influence of the maternal-neonatal interface on the gut microbiota in autoimmune disease is a poorly understood, further research is required regarding the impact of maternal probiotics on the development of the fetus and the implications in autoimmune disease. A number of gaps exist in the literature regarding maternal probiotics for autoimmune diseases in both mothers and their children. Designing studies aimed at understanding how probiotics act in an immunomodulatory capacity to ameliorate or exacerbate autoimmune diseases in pregnancy and infancy is, without excuse, a requisite edge for cutting into the issues surrounding autoimmunity and pregnancy.

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REVIEW
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Exploring the Human Microbiome: The Potential Future Role of Next-Generation Sequencing in Disease Diagnosis and Treatment

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The interaction between the human microbiome and immune system has an effect on several human metabolic functions and impacts our well-being. Additionally, the interaction between humans and microbes can also play a key role in determining the wellness or disease status of the human body. Dysbiosis is related to a plethora of diseases, including skin, inflammatory, metabolic, and neurological disorders. A better understanding of the host-microbe interaction is essential for determining the diagnosis and appropriate treatment of these ailments. The significance of the microbiome on host health has led to the emergence of new therapeutic approaches focused on the prescribed manipulation of the host microbiome, either by removing harmful taxa or reinstating missing beneficial taxa and the functional roles they perform. Culturing large numbers of microbial taxa in the laboratory is problematic at best, if not impossible. Consequently, this makes it very difficult to comprehensively catalog the individual members comprising a specific microbiome, as well as understanding how microbial communities function and influence host-pathogen interactions. Recent advances in sequencing technologies and computational tools have allowed an increasing number of metagenomic studies to be performed. These studies have provided key insights into the human microbiome and a host of other microbial communities in other environments. In the present review, the role of the microbiome as a therapeutic agent and its significance in human health and disease is discussed. Advances in high-throughput sequencing technologies for surveying host-microbe interactions are also discussed. Additionally, the correlation between the composition of the microbiome and infectious diseases as described in previously reported studies is covered as well. Lastly, recent advances in state-of-the-art bioinformatics software, workflows, and applications for analysing metagenomic data are summarized.

Keywords: microbes, human microbiome, host-microbe interactions, metagenomics, next generation sequencing, bioinformatics, dysbiosis, diseases

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INTRODUCTION

Microbes are ubiquitous in nature, inhabiting almost every conceivable environment, and play an important role in human life. Microbes, though generally invisible, play an essential role in ecosystem functioning (1, 2), modulating key ecosystem processes such as plant growth, soil nutrient cycling, and marine biogeochemical cycling (3–6). An innumerable number of symbiotic, pathogenic, and commensal microbes colonized the human body; collectively constituting the human microbiota. Interactions between the human body and gut-microbiota are widely recognized as influencing several aspects of human health (7). A functioning microbiome is obligatory for host organisms, as it contributes to the smooth functioning of important physiological processes. In fact, host organisms have co-evolved with their microbiota; with some commensals having evolved as pathobionts while others as symbionts (8, 9). The presence of certain commensals in the human gut induces signals that drive proper functioning and maturation of the immune system. Microbial communities take on a specific structure within different hosts and physical environments (10). Consequently, identification and characterization of the microbes inhabiting a host, their distinct host phenotypes, and the biochemical pathways by which microbes impact their hosts are the major focus of host-microbiome research.

Analyses of host-microbe interactions can reveal the core characteristics of the interaction, including their identification, classification, profile prediction, and mechanisms of interaction. Although the structure, function, dynamics, and interactions of these microorganisms play an essential role in human metabolism; their identification, quantification and characterization can be problematic. The majority of microbial communities are extremely diverse and most of the individual organisms have not yet been cultured (11). Secondly, their interaction with each other and tendency to form intricate networks makes it difficult to predict their behavior (3). Establishing mechanistic connections between gut-microbiota and its functioning adds an extra challenge especially in understanding the biology of intricate microbial consortia (12). Classic approaches to microbial ecology have relied on cultivation-dependent techniques to study host-microbe interactions. Although these culture-dependent techniques have generated interesting data sets, they have also resulted in a spurious view of microbiota. Recently, however, a number of culture-independent techniques, mainly PCR-based methods, have evolved for the qualitative and quantitative identification of microbes. These techniques have entirely changed the perception of the human microbiome and have paved the way for the establishment of metagenomics. Metagenomic studies are increasing our knowledge of host-pathogenic interactions by revealing the genes that potentially allow microbes to influence their hosts in unexpected ways. Metagenomic studies of host-microbe interactions can provide useful information applicable to a wide array of disciplines; including pathogen surveillance, biotechnology, host-microbe interactions, functional dysbiosis, and evolutionary biology (13). Recent studies of host microbiomes using metagenomic approaches have offered key insights into host-microbe interactions.

In addition to allowing researchers to characterize the composition of microbial communities, metagenomic studies have also provided novel information on other aspects of the biological sciences. For example, metagenomic studies on the human microbiome have revealed possible links between the gut microbiome and human diseases such as depression (14), rheumatoid arthritis (15) and diabetes (16). Several studies have utilized materials from ancient communities to trace changes in the microbiome. These studies have conducted metagenomic studies of coprolites (17), teeth (18), and other tissues (19). Provided that nucleic acids can be extracted from the sample, almost any material from an environment can be used in metagenomic analyses. One of the largest metagenomic studies to date is the Global Ocean Sampling. Metagenomics is also being applied to the field of medicine. **Figure 1** illustrates the timeline of sequence-based metagenomic studies and shows the range of environments that have been sampled and analyzed between 2003 and 2017. Several articles have been published that have focused on metagenomic methodology and analysis software (20–27). The present review attempts to provide an overview of the high-throughput sequencing technologies and analytical software currently available for studying host-microbe interactions. Moreover, there is an attempt to also highlight the advancement of sequencing techniques over time and provide information regarding the appropriateness for applications in exploring the human microbiome and the metagenomes of other diverse environments. Lastly, a discussion is provided of the various bioinformatic options that are available to successfully meet both *de novo* sequencing and sequence alignment challenges.

UNSEEN MICROBIAL DIVERSITY AND ITS GLOBAL IMPLICATIONS

Microbes conduct significant functions that greatly benefit the health of planet, as well as its inhabitants. Microbes help to regulate, modulate, and maintain earth's atmosphere (28), support the growth of plants and help to suppress plant diseases (29), contribute to human health (30), breakdown harmful chemicals present in contaminated environments (31, 32), support sustainable farming (33), modulate greenhouse gases (34), are primary components of various biogeochemical cycles (35) and greatly contribute to ecological processes, including climate change (36). In addition to remediating contaminated environments and modulating the atmosphere, the combined

Abbreviations: PCR, Polymerase chain reaction; NGS, Next generation sequencing; GIT, Gastrointestinal tract; IBD, Inflammatory bowel disease; IBS, Irritable bowel syndrome; SCFA, Short-chain fatty acids; PYY, Peptide YY; HIV, Human immunodeficiency virus; KEGG, Kyoto Encyclopaedia of genes and genomes; GOLD, Genomes Online Database; SBS, Sequencing by synthesis; dNTP, Deoxyribonucleotide triphosphate; PGM, Personal genome machine; ISFET, Ion-sensitive, field-effect transistor; SMRT, Single-molecule, real-time sequencing; ZMW, Zero mode wave; ONT, Oxford Nanopore Technologies; MG-RAST, Metagenomics Rapid Annotation using Subsystem Technology; EBI, European Bioinformatics Institute; QIIME, Quantitative Insights Into Microbial Ecology.

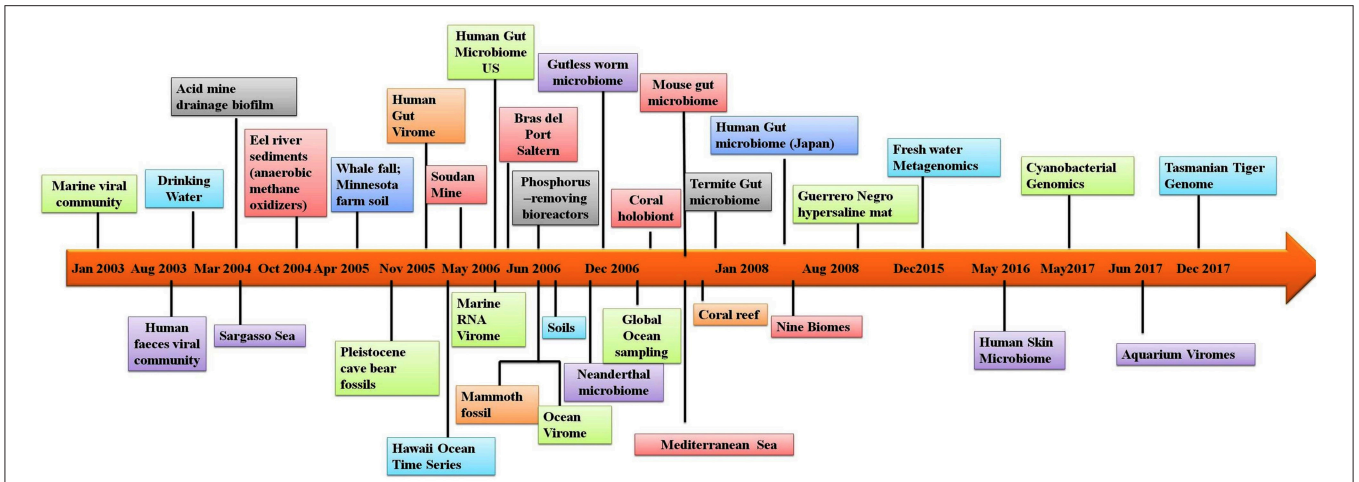


FIGURE 1 | Timeline of the sequence-based metagenomic projects showing the variety of the environmental samples.

activity of these invisible microbial communities shape the face of the biosphere and represent untapped reservoirs of novel biomolecules; including pharmacological drugs and industrial enzymes (37). Microbes coexisting in the human body offer a variety of benefits by modulating fundamental metabolic processes, immunity, and signal transduction. Increasing evidence suggests that there is a significant association between the human gut microbiome and the development of human diseases (38).

Previously, it was difficult to study microbes in their natural environment and thus microbiologists were limited to studying individual species in the laboratory. This approach, however, has limited the data that can be obtained on microbial communities inhabiting diverse ecosystems. Metagenomics has helped to resolve this limitation and has greatly increased our understanding of entire microbial communities, thus significantly advancing our knowledge of microbial ecology and microbiology in general. Metagenomics, supported by next-generation-sequencing (NGS) has literally removed the limitations and boundaries associated with classic culture-based approaches (39–41). NGS technology has enabled the comprehensive study of diverse microbiomes in their native environments, including the ocean microbiome (42), human skin microbiome (43), human microbiome (44) and the Saragossa Sea microbiome (45). Some of the novel findings enabled by metagenomics involve the identification of endosymbiotic bacterial phyla (46), nitrification processes (47, 48), human disease pathogens associated with epidemics (49), bacteria (50), and viruses (51) associated with inflammatory bowel diseases, and the identification of commensal gut bacteria (52).

MICROBIOME IN HUMAN HEALTH AND DISEASE: A MECHANISTIC LINK

The human body serves as a host to a networked community of microorganisms (microbiome) that outnumber the body's own cells. Research on the human microbiome has been the area of immense interest over the past few years due to intimate linkage of the microbiome with human health. The human microbiome "our second genome" has intimately co-evolved with humans for millions of years and plays a critical role in human health. Deciphering the composition and function of the human microbiome can provide a deeper understanding of its' structural and functional properties. In the future, our understanding of the human microbiome and the application of metagenomic analyses will greatly enhance our understanding of human health and disease in specific individuals. The exploration of human microbiome and metagenome is considered to represent a frontier in human genetics.

The majority of research on the human microbiome has focused on the microbes colonizing the human digestive system, as these microorganisms are believed to influence human health in a number of ways. The digestive system microbiome is extremely diverse, with significant variations in its constituents across individuals (44). Modulation of the microbiome by extraneous factors, such as fecal transplantation and dietary intervention, has been shown to be a potential therapeutic approach to addressing a number of health-related problems (53). The gastrointestinal tract (GIT) harbors a vast diversity of microbes, comprising the intrinsic networks of both microbe-microbe and host-microbe interactions (54). Microbial guilds (species that exploit the same resources) have been found to provide an interesting feature that can be used to help understand processes taking place at both a single cell and community level. Microbes under normal physiological conditions are commensal and mediate digestion, strengthen the immune system and inhibit or prevent pathogens from invading the body. The linkage between the human microbiome and human health remains largely unknown and unexplored, however, a number of epidemiological studies have found that the overall reduction in the diversity of digestive system microbiota is linked to diseases such as eczema (55), asthma and inflammatory diseases (56), diabetes and obesity (57), allergies (58), digestive tract disorders such as IBD (inflammatory bowel disease) (59)

and IBS (irritable bowel syndrome) (60). Dysbiosis (microbial imbalance) has also been linked with the genesis and evolution of a plethora of other diseases, including chronic fatigue syndrome (61), cancer (62), colitis (63) bacterial vaginosis (64), and anxiety and depression (65). Several recent studies have highlighted the critical role that the gut microbiome plays in modulating different immune responses, including immune tolerance, *via* Treg (T regulatory) cell modulation. Studies carried out by Geuking et al. (66), indicated that short-chain fatty acids (SCFA) can promote the development of Treg cells in the gut. Gut-inhabiting microbes facilitate the breakdown of complex carbohydrate (67) and help in the utilization of polysaccharides (68). Other examples of the health-supporting functions of the gut-microbiome are protection against diseases *via* immune modulation (69), fecal microbiome transplantation (70), metabolism, xenobiotic toxicity and pharmacokinetics (71).

THE MICROBIOME AS A THERAPEUTIC AGENT

As mentioned, the human body is teeming with trillions of microbes, collectively called the "human microbiome." Microbiome studies have now become a prominent field of research by offering potential and novel methods of disease diagnosis, prognosis, and treatment. Microbial ecology within an ecosystem involves a cross-talk among its inhabitants. The growth and survival of microbes in any ecosystem are largely governed by their chemical environments, and microbes have evolved the ability to adapt and utilize different chemicals through specific genes (72, 73). Alterations (good and bad) in the microbial equilibrium of the gut microbiome do occur. Science has developed medications that have a significant impact on the microbial equilibrium. Beneficial microbes colonizing the gut produce a variety of chemicals, including analgesics, vitamins, antioxidants and anti-inflammatory factors that protect and support the normal functioning of the human body. Dysbiosis (disruptions in microbiota) has been associated with different diseases. Therefore, maintaining a beneficial gut microbiome, in terms of both composition and function, is important for human health (74, 75). The gut microbiome has an active relationship with its human host and exhibits a regulatory role in cognition, mood, pain, and anxiety, exerted through a gut-brain axis. Drastic changes in the maternal microbiome that occur during pregnancy influence the maturation and immunity of neonates. Studies carried out by Ng et al. (76) indicated that increased levels of salicylic acid in the intestines contribute to the proliferation of pathogenic bacteria in the GIT when patients are treated with antibiotics. Roberfroid et al. (77) reported that the consumption of prebiotics (indigestible plant fiber) induces specific changes in the gut microbiome, elevating levels of SCFA (short chain fatty acid). Studies reported by Cani et al. (78) stated that fermentation activity carried out by the gut microbiome results in reduced hunger and increased satiety levels, which as a result, decreases total energy inputs. Similarly, studies carried out by Archer et al. (79) and Whelan et al. (80) confirmed that fermentation of non-digestible carbohydrates by the gut microbiome controls food intake activity and reduces energy intake. According to Parnell et al. (81), prebiotic-induced changes in the gut microbiome of obese patients decreases the circulation of lenomorelin or ghrelin (a hunger hormone) and increases the peptide, tyrosine or PYY. In contrast, however, studies carried out by Peters et al. (82) and Hess et al. (83) indicated that prebiotic treatments do not influence the appetite. A recent study by Tarini et al. (84) demonstrated that a single dose of insulin significantly decreases levels of lenomorelin blood plasma and augments post-prandial plasma levels of Glucagon-like peptide-1. In short, there is a growing body of evidence on the contribution of the microbiome on human health and increased understanding that the microbiome can serve as a potential therapeutic agent.

DISSECTING THE HOST-PATHOGEN MICROBIOME

Host-pathogen interactions have profound consequences in human biology and can be viewed as a battle between two systems. Pathogens, which are the invaders, can seize host cells and use them for their own advantage (8), and they can evolve so quickly that they overpower the human immune system, as with HIV infection (85). The conflict between the interacting partners results in phenotypic changes and is believed to be the main driving force for a number of phenomena, such as speciation and the evolution of sex (86). Detailed mechanistic analyses of host-pathogen interactions are varied with most still in need of further study. Notably, little is known about the molecular level dynamics of host-pathogen interactions and the need for more studies on this topic are critical, especially those dealing with the molecular events that regulate phenotypic changes in the host. Advancements in Next Generation Sequencing (NGS) technologies and bioinformatic tools have offered new approaches for studying host-pathogen systems. Researchers are now able to construct the genomes of both model and non-model organisms. The use of these newly-developed tools allows researchers to not only study the behavior of a single gene under different conditions but also study the extensive impact of these host-pathogen interactions on molecular environments (global gene expression). Several open-source, standalone R packages and web-based software programs have been developed to help and acquire key insights in understanding the host-pathogen microbiome (**Figure 2**). A more detailed account of metagenomic software and resources are given in a separate section of this review wherein we mentioned some of the standard software used for quality control, taxonomic classification, diversity metrics, annotation and functional information, sequence classification, metabolic pathway reconstruction, and statistical analyses.

MICROBIAL CENSUS

Culture-independent methods are the most appropriate for ascertaining the abundance of microbes that are present within

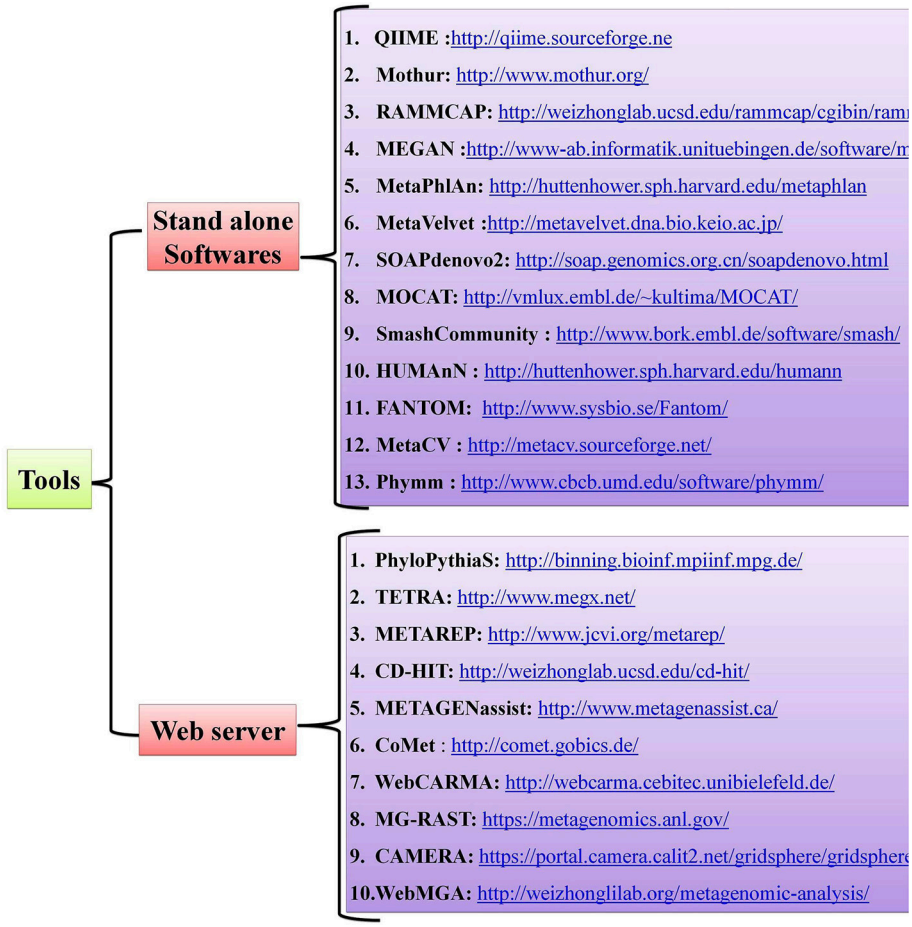


FIGURE 2 | Tools and web servers related to gut microbiome studies.

a community. DNA re-association kinetics provide information on both community structure and diversity (87). 16S rRNA gene sequencing is one of the main methods used for identifying the microbial taxa present in a community (88). The utility of this approach is based on the fact that the DNA sequence of regions between conserved areas of 16S rRNA vary among different bacterial species and can be species specific. Two different sequencing approaches used for studying microbial communities are (i) the targeted sequencing (16Sr RNA) and (ii) shotgun sequencing of the metagenome. Each of these methods can provide strikingly different results when used in metagenomic analyses. Shotgun sequencing methods are generally considered superior for the identification and characterization of microbial communities, as they typically provide a greater level of diversity compared to amplicon sequencing (89). Amplicon-based sequencing matches the DNA sequence amplified using a set of universal primers based on the highly-conserved 16S rRNA to sequences of known bacterial taxa. In contrast to amplicon sequencing, shotgun sequencing engages a genome-wide approach, utilizing random strings of genomic DNA sequences obtained by breaking total genomic DNA and matching the obtained sequences to an annotated database of known DNA sequences using clade-specific marker genes or common sequences. Shotgun metagenomics is often used for gene cataloging and functional inference (10). Deep sequencing of metagenomic samples, as was used in the Human Microbiome Project and Metagenomics of the Human Intestinal Tract, provides extensive sequence information even of minor components (taxa) present in the metagenome. This allows for the identification and characterization of the genes present within a given microbial community. The obtained sequences reads can either be used directly or first assembled into contigs, which are then compared to an available database for the identification of specific genes. *De novo* gene prediction is also possible (90), which may identify motifs with functional inference. Gene catalogs can also be compared with databases such as KEGG (the Kyoto Encyclopedia of Genes and Genomes) (91), which arranges the gene products into biological processes and pathways (**Figure 3**).

METAGENOMICS AND MICROBIAL STUDIES

Metagenomics is expected to play a major role in advancing our understanding of microbes and microbial communities.

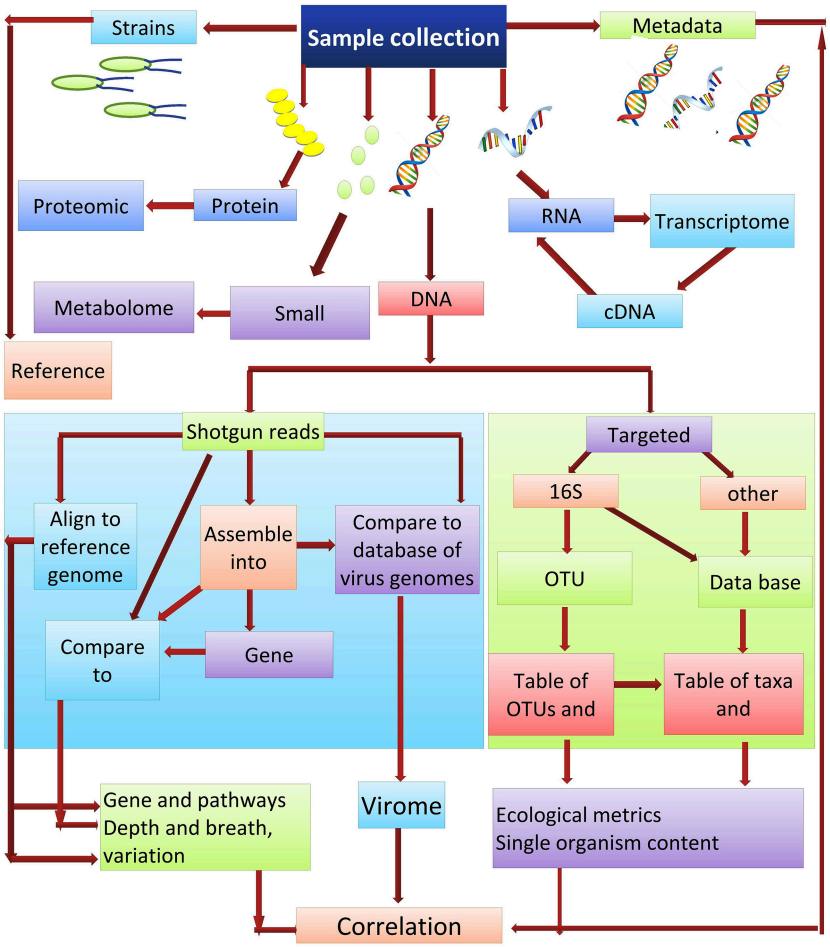


FIGURE 3 | Microbiome analysis workflow.

It is tempting to suggest that metagenomics can serve as a "universal test" for pathogens, eliminating the need to perform lengthy serial testing involving specific assays. Recent advances in sequencing techniques allow almost the entire genome of individual microorganisms to be assembled directly from environmental samples. Metagenomic analyses are playing a decisive role in the characterization of human microbial communities, as well as in determining the relationship between the resident microbiome and invasive pathogens. The accumulation of sequencing data has enhanced our recognition and understanding of the changing nature of microbial populations and their impact on the environment (92) and on human health (93). Metagenomics is not only helping to identify and characterize the human gut microbiome but is also identifying novel genes and microbial pathways, as well as functional dysbiosis. Clearly, metagenomics has become an indispensable and fast-growing discipline in modern science. Advances in NGS has led to a substantial increase in the number of metagenomic studies listed in the Genomes Online Database (GOLD) (https://gold.jgi.doe.gov). These studies span a broad environmental spectrum, including natural communities; as well as engineered and clinical environments (94, 95).

STUDY OF MICROBIOME PRIOR TO NGS

Prior to the advent of NGS technologies, the accurate profiling of microbial communities was challenging. The same was true for characterizing the human gut microbiome, a highly dense and diverse community containing only a small proportion of microbes that could be cultured (96). Early studies of the human gut microbiome involved the culturing of the microbes present in samples (97) and studying the interactions between co-cultured microbial taxa (98). These techniques, however, provided information on only a limited set of microbial taxa and microbial interactions. They failed to provide information about the composition of the entire community and the dynamics occurring between the taxa comprising the total community. The emergence of NGS technologies has overcome the limitations characteristic of studies based on culturing techniques.

DECIPHERING HOST-PATHOGEN INTERACTIONS IN THE ERA OF NGS TECHNOLOGIES

The advent of NGS technologies have greatly enhanced the ability to identify and characterize metabolic and regulatory mechanisms through which hosts and microbes interact with each other to define a healthy or diseased state in the host organism. NGS technologies are invaluable for the exploration of the composition of the microbiome and exploring the genetic, functional, and metabolic properties of the microbial community. Sanger sequencing (99), the first generation of DNA sequencing technology, was one of the widely used sequencing method for more than three decades and is still used today for low-throughput DNA sequencing or sequencing of single DNA entities. Sanger DNA sequencing is based on the principle of the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase. This technique was the major approach used in the Human Genome Project in 2001. The high cost of Sanger sequencing and volume (number of sequences) limitations reduced its potential for high-throughput sequencing.

Exploring Host-Pathogen Interactions

Advances in NGS technologies now provide a fast, cost-effective approach to delivering large volumes of highly-accurate data that has resulted in a major paradigm shift over the past few decades (100, 101). Time and cost were originally the main stumbling blocks associated with sequencing technology. The advantages of NGS over classic Sanger sequencing are that it is cost-effective, devoid of a cloning step, offers highthroughput, and requires minimal technical expertise. A major challenge with NGS data, however, is the analysis of millions of sequences that allows one to achieve statistically and scientifically meaningful conclusions (**Table 1**).

Several different NGS platforms have been developed (**Figure 4**) and are commonly used. These include the Roche 454 GS FLX, Illumina (MiSeq and HiSeq), Ion Torrent/IonProton/Ion Proton, SOLiD 5500 series, and Oxford Nanopore. At present, the majority of microbial studies using high-throughput sequencing have focused on either functional metagenomics (103) or amplicon sequencing (104).

Roche 454 Genome Sequencer

This sequencing platform is based on the principle of pyrophosphate release, which was originally described by Nyrén et al. (105) in 1985 and reported by Hyman (106) in 1988. Roche 454 was produced and made commercially available in 2005, and advertised as the first available high-throughput sequencing system. The system utilizes sequencing by synthesis (SBS), in which adapters are ligated to DNA fragments that cause the binding of the fragments to microbeads in a Pico Titer Plate (https://www.roche.com/). Amplification of the DNA fragments is carried out by Emulsion PCR, in which water droplets containing a single bead and PCR reagents are immersed in oil. The long read length (400–500 bases with paired-ends), along with its high efficiency, were more advantageous than what other NGS platforms could provide at that time; and thus was used for genome sequencing. The system generates 20 Mb of sequences per run with an average read length of approximately 100 bp (107). One of the notable applications of the Roche 454 system was the identification of the agents responsible for the epidemic disease of honeybees (108). Additional information about Roche 454 Genome Sequencer can be obtained at http://www.roche.com.

Illumina Genome Sequencer

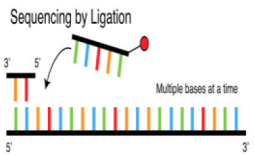
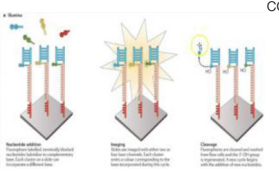
The Illumina sequencing platform first emerged in 2006, and was followed by the acquisition of Solexa by Illumina in 2007. Illumina possesses an array of the most commonly sequencing systems and has rapidly been adopted by many researchers throughout the world. This is due to its' cost-effectiveness, and longer read length (although a limitation in the earlier version of the Illumina, which was subsequently improved in the newer version, MiSeq 2 × 300 bp). This led to a major shift by the scientific community from using the Roche 454 platform to Illumina technology (109). Illumina follows the principle of SBS chemistry, by incorporating reversible chain terminator nucleotides for all four bases, the labeling of each base with a different fluorescent dye, and the use of a DNA polymerase (110). Sequencing involves the ligation of specific adapters to both ends of short DNA fragments, and the immobilization of one of the adapters by binding to a solid support. The adapters hybridize with specific oligonucleotides bound to a proprietary substrate within a micro fluid flow cell. Fluorophore-bearing nucleotides are then introduced one by one and incorporated into the growing complementary strand by a DNA polymerase. Sequential images are captured and analyzed to identify the nucleotide that is incorporated in the growing strand and the cycle is repeated with different nucleotide species. The resulting reads have a final length of 35 nucleotides (111).

Illumina, however, introduced an upgraded version of their technology, the Genome Analyser II, which tripled the output relative to earlier versions of the Genome Analyser. Presently, the IlluminaMiSeq offers one of the longest (300 bp) read lengths of all of the Illumina products; facilitating the sequencing of paired-end reads (104). Another Illumina platform, the Illumina HiSeq, however, is able to generate approximately 200 Gbp of sequences with a single read of 2 × 100 bp (paired-end) per run (112). Additional information about the various Illumina sequencers can be obtained at http://solexaqa.sourceforge.net/ (113).

Qiagen Gene Reader

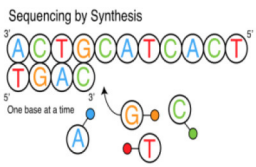
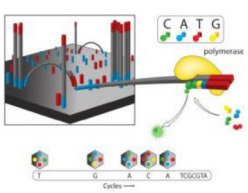
In 2012, Qiagen introduced the Intelligent BioSystems cyclic reversible termination platform, which was commercialized in 2015 under the name Gene Reader (114). In contrast to other next-generation platforms, the Qiagen Gene Reader is the first all-in-one platform that can execute all of the steps required for sequencing DNA, from sample preparation to analysis. To achieve this goal, the Gene Reader sequencer was combined with the QIA cube sample preparation system and the Qiagen Clinical Insight platform for variant analysis. Gene Reader virtually utilizes the same approach as Illumina, apart from the fact that only a small fraction of the added nucleotides incorporate

TABLE 1 | Advantages and limitations of available Next generation sequencing (NGS) platforms.

Sequencing reaction	Limitation	Advantages	Instruments	Read length in base pairs (bp)	Throughput	Total number of reads	Runtime
Sequencing by ligation or SOLiD sequencing 	This sequencing method has been reported to have problems in sequencing particularly palindromic sequences and relatively slower than other methods.	Relatively cheap	SOLiD 5500 Wildfire	50 (SES)	80 Gb	~700 M^{+}	6 days
				75 (SES)	120 Gb		
				50 (SES)$^{+}$	160 Gb		
			SOLiD 5500xl	50 (SES)	160 Gb	~1.4 bn^{+}	10 days^{+}
				75 (SES)	240 Gb		
				50 (SES)$^{+}$	320 Gb		
			BGISEQ-500 FCS155*	50–100 (SES/PES)$^{+}$	8–40 Gb^{+}	NA†	24 h
			BGISEQ-500 FCL155	50–100 (SES/PES)$^{+}$	40–200 Gb*	NA†	24 h^{+}
Sequencing by synthesis: CRT 	Equipment are very expensive. Requires high concentration of DNA.	Potential for high sequencing yield, depending upon sequencer model and desired application	Illumina MiniSeq Mid Output	150 (SES)$^{+}$	2.1–2.4 Gb^{+}	14–16 M^{+}	17 h^{+}
			Illumina MiniSeq High output	75 (SES)	1.6–1.8 Gb	22–25 M(SES)$^{+}$	7 h
				75 (PES)	3.3–3.7 Gb	44– 50 M(PES)$^{+}$	13 h
				150 (PES)$^{+}$	6.6–7.5 Gb^{+}		24 h^{+}
			Illumina MiSeq v2	36 (SES)	540–610 Mb	12–15M (SES)	4 h
				25 (PES)	750–850 Mb	24–30 M (PES)$^{+}$	5.5 h
				150 (PES)	4.5–5.1 Gb		24 h
				250 (PES)*	7.5–8.5 Gb^{+}		39 h
			Illumina MiSeq v3	75 (PES)	3.3–3.8 Gb	44–50 M (PES)$^{+}$	21–56 h^{+}
				300 (PES)$^{+}$	13.2–15 Gb^{+}		
			Illumina NextSeq 500/550 Mid output	75 (PES)	16–20 Gb	Up to 260 M (PES)$^{+}$	15 h
				150 (PES)$^{+}$	32–40 Gb^{+}		26 h^{+}
			Illumina NextSeq 500/550 High output	75 (SES)	25–30 Gb	400 M(SES)$^{+}$	11 h
				75 (PES)	50–60 Gb	800 M(PES)$^{+}$	18 h
				150 (PES)$^{+}$	100–120 Gb^{+}		29 h^{+}
			Illumina HiSeq2500v2 Rapid run	36 (SES)	9–11 Gb	300 M(SES)$^{+}$	7 h
				50 (PES)	25–30 Gb	600 M(PES)$^{+}$	16 h
				100 (PES)	50–60 Gb		27 h
				150 (PES)	75–90 Gb		40 h
				250 (PES)$^{+}$	125–150 Gb^{+}		60 h^{+}
			Illumina HiSeq2500 v3	36 (SES)	47–52 Gb	1.5 bn (SE)	2 days
				50 (PES)	135–150 Gb	3 bn(PES)$^{+}$	5.5 days
				100 (PES)+	270–300 Gb		11 days^{+}

(Continued)

TABLE 1 | Continued

Sequencing reaction	Limitation	Advantages	Instruments	Read length in base pairs (bp)	Throughput	Total number of reads	Runtime
			Illumina HiSeq2500 v4	36 (SES)	64–72 Gb	2 bbn(SES)	29 h
				50 (PES)	180–200 Gb	4 B (PES)+	2.5 days
				100 (PES)	360–400 Gb		5 days
				125 (PES)+	450–500 Gb+		6 days
			Illumina HiSeq 3000/4000	50 (SES)	105–125 Gb	2.5 bn (SES)+	1–3.5 days+
				75 (PES)	325–375 Gb		
				150 (PES)+	650–750 Gb+		
			Illumina HiseqX	150 (PES)+	800–900 Gb per flow cell*	2.6–3 bn (PES)+	<3 days+
			Qiagen Gene Reader	NA†	12 genes; 1,250 mutations	NA†	Several days
Sequencing by synthesis: SBS 	Homopolymer errors	Less expensive and relatively fast	454 GS Junior	Upto 600;400 average (SES,PES)*	35 Mb+	~ 0.1 M+	10 h+
			454 GS Junior+	Upto 1,000;700 average (SES,PES)+	70 Mb+	~ 0.1 M+	18 h+
			454GSFLX TitaniumXLR70	Upto 600;450 mode (SES,PES)+	450 Mb+	~1 M*	10 h+
			454 GS FLX Titanium XL+	Up to 1,000; 700 mode (SE, PE)+	700 Mb+	~1 M+	23 h+
			Ion PGM 314	200 (SES)	30–50	400,000–	23 h
				400 (SES)	60–100 Mb+	550,000+	3.7 h+
			Ion PGM 316	200 (SES)	300–500 Mb	2–3 M+	3 h
				400 (SES)+	600 Mb–1 Gb+		4.9 h+
			Ion PGM 318	200 (SES)	600 Mb–1 Gb	4–5.5 M+	4 h
				400 (SES)+	1–2 Gb+		7.3 h+
			Ion Proton	Up to 200 (SES)	Up to 10 Gb+	60–80 M+	2–4 h+
			Ion S5 520	200 (SES)	600 Mb–1 Gb	3–5 M+	2.5 h
				400 (SES)+	1–2 Gb+		4 h+
			Ion S5 530	200 (SES)	3–4 Gb	15–20 M+	2.5 h
				400 (SES)+	6–8 Gb+		4 h+
			Ion S5 540	200 (SES)+	10–15 Gb+	60–80 M+	2.5 h+
Single-molecule real-time long reads or (Pacific BioSciences) 	Moderate throughput and equipment are very expensive	Fast detection	Pacific BioSciences RSII	~20 Kb	500 Mb–1 Gb+	~55,000+	4 h+
			Pacific BioSciences Sequel	8–12 Kb	3.5–7 Gb+	~350,000+	0.5–6 h+
			Oxford Nanopore MK1MinION	Up to 200 Kb	Up to 1.5 Gb	>100,000	Up to 48 h
			Oxford Nanopore PromethION	NA†	Upto 4 Tb+	NA†	NA†

*+Manufacturer's data; *Rounded from Field Guide to next-generation DNA sequencers and 2014 update; †Information is not available, as this product has been developed recently. CRT, Cyclic Reversible Termination; NA, Not Available; PES, Paired End Sequencing; SBS, Sequencing by Synthesis; SES, Single End Sequencing.*

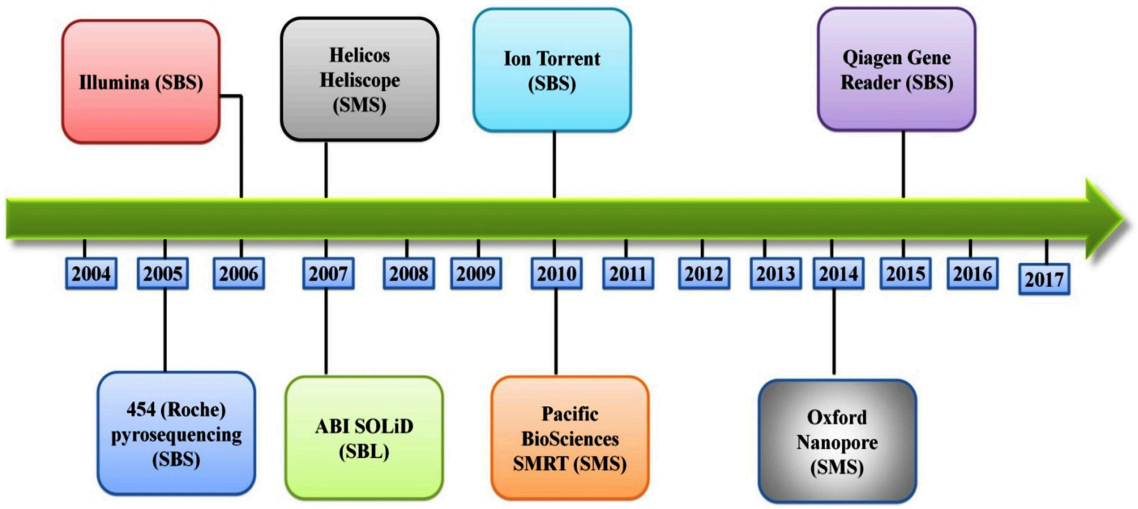


FIGURE 4 | Timeline of the introduction of the next-generation DNA sequencing technologies and platforms.

fluorophore-labeled dNTP (115). Qiagen's Gene Reader usually runs up to four flow cells at a time, with each flow cell running up to ten samples. The flow cells can be added in mid-run via a "turntable" within the instrument. Additional information on the Qiagen Gene reader can be obtained at http://www.qiagen.com.

ABI SOLiD (Sequencing by Oligonucleotide Ligation and Detection) System

Applied Biosystems, through the Life Technology subsidiary, introduced the SOLiD platform in 2007. The system employs a unique chemistry for sequencing by ligating oligonucleotide adapters to DNA fragments and immobilizing the ligation products on beads, which are then placed on a water-oil emulsion (116). The beads on which DNA amplification occurs are deposited on glass slides and subjected to sequential hybridization with a universal PCR primer complementary to the adapters. The ligation step is then followed by fluorescence detection.

Ion Torrent Sequencing Technology (PGM, Proton, S5 Series)

Ion Torrent introduced the personal genome machine (PGM) in 2010 as a cost effective platform for DNA sequencing (117). Unlike other sequencing technology, Ion Torrent does not make use of optical signals (118) but rather utilizes an enzymatic cascade to generate a signal. The Ion Torrent system utilizes high-density micro-machined wells to carry out nucleotide additions in a massively parallel approach. Each micro-well contains a different DNA template. There is an ion-sensitive layer and an ion-sensor located under each well. The technology works on the principle of detecting the proton (H^+) released during the incorporation of each dNTP in a growing DNA template. The release of H^+ ion results in a change in pH that is detected by an integrated ion-sensitive, field-effect transistor (ISFET) (117). In the case of two identical bases, the output voltage is doubled. Ion torrent platform can generate upto 10 Gb of sequence data in a single run, with a maximum of 50 million reads having an average read length of 200 bases. The PGM can also provide 5.5 million reads having an average read length of 400 bp, producing a maximum of 2 Gb of sequence data from 318 V2 chip. A notable aspect of this technology is the size-selection step in which sequencing of longer fragments is omitted (https://www.thermofisher.com/in/en/home/brands/ion-torrent.html). Additional information about Ion Torrent technology can be obtained from https://www.thermofisher.com/in/en/home/brands/ion-torrent.html.

THE THIRD GENERATION OF SEQUENCING TECHNOLOGY

At present, the described sequencing technologies are the most commonly used for metagenome projects, however, sequencing technologies have undergone rapid advances during the past few years to attempt to resolve the biases associated with the current methods and to obtain a better balance between data yield, read length, and cost. These efforts have resulted in third generation sequencing technologies, such as Oxford Nanopore (119), and PacBiosequencing platforms (120) which are single-molecule and real-time technologies that reduce amplification bias, as well as short read length problems. The reduction in the cost and time presented by these sequencing methods are valuable asset. Although the error rate with the newer technologies is much higher relative to the other described sequencing technologies, this problem can be addressed by increasing the sequencing depth.

Pacific Biosciences

Pacific Biosciences established the first DNA sequencer that utilizes a single-molecule, real-time sequencing (SMRT) approach. This sequencing platform has become one of the most

widely used third-generation sequencing technologies (121). The platform is based on the sequencing by synthesis principle. Pacific Biosciences makes use of the same fluorescent dyes as other NGS technologies, however, instead of carrying out the cycles of nucleotide amplification in the same manner as other sequencing technologies, the signals emitted upon the incorporation of the nucleotides are detected in real time. Sequencing is carried out on a chip (SMRT cell) that contains several zero mode wave (ZMW) guides. A single DNA polymerase is immobilized to the bottom of each ZMW guide with a molecule of single stranded DNA template (122). Four phospholinked nucleotides, each labeled with a different fluorescent dye producing a distinct emission spectrum, are also added to SMRT cells. Once the nucleotide is incorporated by the DNA polymerase, a light signal is produced and a base call is made and recorded (122).

Helicos Biosciences

Heliscope was released by Helicos Biosciences in 2007. It is also a single-molecule sequencing device. Sequencing is carried out in a glass flow cell with 25 channels for samples. The samples can either be replicates of the same sample or different samples. The Heliscope platform utilizes emulsion PCR amplification of DNA fragments in order to obtain significantly higher signals for reliable base detection by multiple charge-coupled device cameras. Single-molecule sequencing methods have the potential to deliver consistently low error rates by eliminating amplification-related bias, intensity averaging, and synchronization problems (123, 124). In the Heliscope platform, 100–200 oligonucleotide fragments are initially immobilized on a proprietary substrate within a microfluidics flow cell. Fluorescence-labeled nucleotides are then introduced individually and are incorporated by DNA polymerase into the growing complementary strand. The fluorophore-bearing nucleotide increases detectability and eliminates the need for amplification of the DNA template. Images are recorded and analyzed to identify the nucleotide that has been incorporated into the growing strand before the cycle begins with a different fluorescently-labeled nucleotide. At present, the Heliscope can only provide a read length of 35 nucleotides (111). Additional information can be obtained at http://www.helicosbio.com.

Oxford Nanopore Sequencing

Oxford Nanopore Technologies (ONT) is at the forefront of developing nanopore sequencing technology (http://www.nanoporetech.com/). The Nanopore platform does not require an amplification step as a part of library preparation. The novelty of this approach is that the DNA strand to be sequenced can be directly analyzed. Oxford Nanopore Technologies introduced the MinION (125) device in 2014. It has the potential to provide longer reads with better resolution of repeated sequence elements and structural genomic variants (126). MinION is a mobile, single-molecule Nanopore sequencer measuring four inches in length and is connected to a laptop with USB 3.0. Nanopore sequencing technology is based on the principle of modulation of the ionic current as a DNA molecule traverses through the nanopore, revealing characteristics of the molecule such as conformation, length and diameter. The pore consists of a protein within a conductive electrolytic solution which creates a small potential gradient across the protein pore (127). MinION mk1B is a pocket-sized portable sequencing device, containing 512 nanopore channels, and can be directly linked to a computer for data collection. More recently, a more advanced device, "PromethION," has been commercialized (127). PromethION is a benchtop sequencer possessing 48 individual flow cells, each consisting of 3,000 pores that are equivalent to 48 MinIONs processing 500 bp/s (128). The capabilities of this instrument provide sequencing power that is sufficient to conduct sequencing of large genomes, such as the human genome. Additional information on Oxford Nanopore sequencing technologies can be obtained at https://www.nanoporetech.com.

So far, the present review has provided an overview of the first through third generations of sequencing technology that have provided significant improvements in the ability to conduct microbiome research. Metagenomic and other omic approaches are the most effective methods that can be used to characterize microbial communities, as well as their metabolic activity. It is now feasible to obtain information on the composition (taxa), diversity, pathogenesis, evolution, and drug resistance of microbes. The selection of any of the above mentioned platforms, however, should be mainly dependent on the aim, design, and purpose of the study. Illumina sequencing technology has made tremendous advances in data output and cost efficiency over the past few years and as a result, presently dominates the NGS market (129, 130). Illumina sequencing technology has been used extensively in numerous microbiome research projects (131–133), including the Human Microbiome Project (44). While both Ion Torrent and Illumina sequencers provide a number of advantages in terms of their cost and efficiency, the short read lengths they provide make them less appropriate for addressing a number of scientific questions, including detection of gene isoforms, methylation detection, and genome assembly (118). SMRT (single-molecule real-time) sequencing platforms offer approaches that are more suited for these research objectives. Since PacBiosequencing generates longer reads that provide longer scaffolds (134–136), it is well suited for *denovo* genome assembly. The commercial availability of MinION sequencers by Oxford Nanopore Technologies, which resemble a USB flash drive in appearance, has also enabled applications that require long-read sequencing (137, 138). The efficiency, long read lengths, and single-base sensitivity make nanopore sequencing technology a promising approach for high-throughput sequencing. The MinION system has been used for sequencing the genomes of infectious agents, including the analysis of bacterial antibiotic resistance islands (137), the influenza virus (139) and genome surveillance of the Ebola virus (140). The advancements in high-throughput sequencing technologies now provide the opportunity to choose different sequencing platforms to conduct microbiome research. In a comparative analysis of the Illumina MiSeq, Ion Torrent PGM, and 454 GS Junior sequencing platforms, Loman et al. (141) reported that Illumina provided the highest output per run

TABLE 2 | Lists of software's used in metagenomics analysis.

Software	Application	Link (website)	References
FastQC	FastQC, a java based application is performed via a series of analysis modules. FastQC can either run in a non-interactive mode or in a standalone interactive mode. FastQC is a quality control tool used for high-throughput sequence data via a series of modular options and giving graphical results of length distribution, quality per base sequence, N numbers, GC content, over representation and duplication.	http://www.bioinformatics.babraham.ac.uk/projects/fastqc/	(175)
Fastx-Toolkit	Fastx is a command based tool kit for the quality control of short-reads and allows processing, format conversion, collapsing and cutting on the basis of sequence identity and length.	http://hannonlab.cshl.edu/fastx_toolkit/index.html	(176)
PRINSEQ	A standalone tool allows integration and analysis into the existing data processing pipelines. PRINSEQ as a tool offers a computational resource that is able to handle huge amount of data generated by next-generation sequencers. It is used for sequences trimming based on in the di-nucleotides occurrences and the sequence duplication (mainly 5′/3′).	http://prinseq.sourceforge.net/	(177)
NGS QC Toolkit	NGS QC Toolkit encompasses user-friendly standalone tools for the quality control of the sequence data generated by next-generation sequencing platforms. The analysis is performed in a parallel environment.	http://www.nipgr.res.in/ngsqctoolkit.html	(178)
Meta-QC-Chain	Meta-QC-Chain is a tool for the quality control analysis performed in parallel environment. Performs mapping against 18S rRNA databases in order to remove the eukaryotic contaminant sequences.	http://www.computationalbioenergy.org/qc-chain.html	(179)
Mothur	Mothur is an open-source, expandable software used for the quality analysis of reads to taxonomic classification, ribosomal gene meta-profiling comparison and calculus of diversity estimators.	http://www.mothur.org/	(180)
QIIME	QIIME pipeline is designed for the task of analyzing microbial communities sampled via a marker gene (16S or 18S rRNA) amplicon sequencing. In its heart pipeline QIIME performs quality pre-treatment of raw-reads, calculate estimates diversity estimates, taxonomic annotation and comparison of metagenomic data.	http://qiime.org/	(181)
MEGAN	MEGAN is a graphical interface tool that allows both taxonomic as well as functional analysis of metagenomic reads. It is based on the BLAST output of short reads and performs comparative metagenomics.	http://ab.inf.uni-tuebingen.de/software/megan/	(20)
CARMA	CARMA provides a clear quantitative and statistical characterization of phylogenetic classification of the reads based on Pfam conserved domains.	http://omictools.com/carma-s1021.html	(182)
PICRUSt	PICRUSt is a tool that serves in the field of metagenomic analysis where the prediction of the metabolic potential is done from the taxonomic information obtained *via* 16S rRNA meta-profiling projects. PICRUSt could be thought of as an automated substitute to manually mining the gene families that are believed to be present in organisms whose sequences are found in a 16S ribosomal RNA.	http://picrust.github.io/picrust/	(183)
TETRA	TERTA is a web-based stand alone program used for the Taxonomic classification and comparison of tetra nucleotide patterns with in a DNA sequence.	http://www.megx.net/tetra	(184)
PhylophytiaS	Composition-based classifier of sequences based on reference genomes signatures	https://omictools.com/pps-tool	(185)
MOCAT	MOCAT is a highly configurable and modular pipeline that includes the quality treatment of metagenomic reads based on single copy marker genes classification and gene-coding prediction. The pipeline makes use of a state-of-the-art program to map quality control and assemble reads from metagenome samples sequenced at a very high depth (several billion base pairs).	http://www.bork.embl.de/mocat/	(186)
Parallel-meta	Parallel-meta is a comprehensive and automotive software package that offers fast data mining and metabolic function across large number of metagenomic datasets. The functional annotation is based on BLAST best hit results.	http://www.computationalbioenergy.org/parallel-meta.html	(187)
MetaclusterTA	MetaclusterTA is a tool used for the Taxonomic annotation that is based on the binning of reads and contigs. Dependent on reference genomes.	http://i.cs.hku.hk/~alse/MetaCluster/	(188)
MaxBin	MaxBin software is used for the unsupervised binning of metagenomic sequences based on an Expectation-maximization algorithm. For user's expediency MaxBin reports genome-related statistics including GC content, genome size and completeness.	http://bowtie-bio.sourceforge.net/index.shtml	(189)
Amphora and Amphora2	Amphora and Amphora2 is used for the Metagenomic phylotyping via single copy phylogenetic marker genes classification.	http://pitgroup.org/amphoranet/	(102, 190)
BWA	BWA is an algorithm used for the mapping of short-low-divergent sequences to large references. It is based on Burrows–Wheeler transform.	http://bio-bwa.sourceforge.net/	(191)

(Continued)

TABLE 2 | Continued

Software	Application	Link (website)	References
Bowtie	Bowtie is a fast short read aligner to long reference sequences based on Burrows–Wheeler transform.	http://bowtiebio.sourceforge.net/index.shtml	(192)
Genometa	Genometa is a graphical interface applied for taxonomic and functional annotation of short-reads metagenomic data.	http://genomics1.mhhannover.de/genometa/	(193)
SOrt-ITEMS	SOrt-Items is a tool used for taxonomic annotation via alignment-based orthology of metagenomic reads.	https://omictools.com/sort-items-tool	(194)
DiScRIBinATE	Taxonomic assignment by BLASTx best hits classification of reads.	https://www.westgrid.ca/support/software/discribinate	(195)
IDBA-UD	IDBA-UD is a *denovo* assembler of metagenomic sequences with uneven depth.	http://i.cs.hku.hk/~alse/hkubrg/projects/idba_ud/	(196)
MetaVelvet	MetaVelvet is a *denovo* assembler of metagenomic short reads.	http://metavelvet.dna.bio.keio.ac.jp/	(197)
RayMeta	RayMeta, a *denovo* assembler of metagenomic reads and taxonomy profiler by Ray Communities.	http://denovoassembler.sourceforge.net/	(198)
MetaGeneMark	MetaGeneMark is a gene coding sequences predictor from metagenomic sequences by heuristic model.	http://exon.gatech.edu/index.html	(199)
GlimmerMG	GlimmerMG is a gene coding sequences predictor from metagenomic sequences by unsupervised clustering.	http://www.cbcb.umd.edu/software/glimmer-mg/	(200)
FragGeneScan	FragGeneScan is a gene coding sequences predictor from short reads.	http://sourceforge.net/projects/fraggenescan/	(201)
CD-HIT	CD-HIT is a tool used for clustering and comparing of sequences of nucleotides or protein.	http://weizhongli-lab.org/cd-hit/	(202)
HMMER3	HMMER3 is a free and commonly used software package for sequence analysis. It is a Hidden Markov based model used to perform sequences alignments. Used for the identification of the homologus nucleotide and protein sequences	http://hmmer.janelia.org/	(203)
BLASTX	Basic local alignment of translated sequences	http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi	(203)
MetaORFA	MetaORFA is applied for the assembly of peptides obtained from predicted ORFs.	Website not available	(204)
MinPath	MinPath is a tool used for reconstruction of pathways from protein family predictions.	http://omics.informatics.indiana.edu/MinPath/	(205)
MetaPath	MetaPath is used for the identification of metabolic pathways that are differentially abundant within the metagenomic samples.	http://metapath.cbcb.umd.edu/	(206)
GhostKOALA	GhostKOALA is KEGG's internal annotator of metagenomes by k-number assignment by GHOSTX searches against a non-redundant database of KEGG genes.	http://www.kegg.jp/ghostkoala/	(207)
RAMMCAP	RAMMCAP is used for the metagenomic functional annotation and data clustering.	http://weizhong-lab.ucsd.edu/rammcap/cgi-bin/rammcap_2d.cgi	(208)
ProViDE	ProViDE is a tool for the analysis of viral diversity in metagenomic samples.	https://omictools.com/provide-tool	(209)
Phyloseq	Phyloseq is a tool-kit for raw reads pre-processing, diversity analysis and graphics production. It is an R, Bioconductor package.	https://joey711.github.io/phyloseq/	(210)
Metagenome Seq	MetagenomeSeq is designed to determine the analysis of differential abundance of 16S rRNA gene in metaprofiling data. It is also designed to address the effects of both under-sampling and normalization of microbial communities on the basis of disease association detection.	http://bioconductor.org/packages/release/bioc/html/metagenomeSeq.html	(211)
Shotgun Functionalize R	Shotgun Functionalize is an R-Package for the functional assessment of metagenomic data. The package includes tools designed for importing, annotating and visualizing metagenomic data generated via high-throughput sequencing.	http://shotgun.math.chalmers.se/	(212)
Galaxy portal	Galaxy portal is a web repository of computational tools that can be run without informatics expertise. It is a graphical interface and free service.	https://usegalaxy.org/	(213)
MG-RAST	MG-RAST an open source web application is used for the automatic phylogenetic and functional analysis of metagenomes. MG-RAST is one of the biggest repositories for metagenomic data. It is a Graphical interface, web portal and free service.	http://metagenomics.anl.gov/	(214)
IMG/M	IMG (Integrated Microbial Genomes) system serves as a community resource for the analysis, functional annotation and phylogenetic distribution of genes and comparative metagenomics. It is a graphical interface, web portal and free in service.	https://img.jgi.doe.gov/	(215)
Phinch	Phinch is an open source, interactive exploratory data visualizing tool intended to alleviate the analysis of meta-omic datasets. The main features of this software are streamlined visualization workflow, sleek user interface, novel exploration of larger datasets. Accessible via web browser.	http://phinch.org	(215)

(Continued)

TABLE 2 | Continued

Software	Application	Link (website)	References
CAMERA	CAMERA is an important tool that aims to bridge the gaps and to develop methods so as to monitor microbial communities of the oceans. CAMERA's databases incorporate both the genomic and metagenomic datasets, metadata, results from the pre computed analysis and softwares that endorse commanding cross-analysis of the environmental metagenomes.	https://omictools.com/camera-2-tool	(216)
Meta Comp	Meta Comp is a graphical inclusive analysis tool that encompasses a series of statistical analysis approaches along with visualized results for comparative analysis of metagenomics as well as other meta-omics data sets. The software has the features to read files generated via different upstream analysis programs. It has also got the features to automatically choose two-group sample test.	http://cqb.pku.edu.cn/ZhuLab/MetaComp/	(216)

(1.6Gb/run, 60Mb/h) and the lowest error rates. In a study comparing different sequencing platforms (Ion Torrent PGM, Illumina MiSeq and HiSeq) for the shotgun sequencing of six human stool samples, Clooney et al. (142) concluded that the best assembly values were obtained using the Illumina HiSeq platform, in which 10 million reads per sample were produced. In contrast, the Illumina MiSeq and Ion Torrent PGM did not produce a sufficient number of reads to produce an adequate genome assembly (143).

CORRELATION BETWEEN THE MICROBIOME AND INFECTIOUS DISEASES

Human gut microbiome signatures exhibit individual specificity. There is a high degree of inter individual variation that is based on both host genetics and environmental factors (144, 145). The high degree of individual specificity, however, has hampered our understanding of function of the gut microbiome and its importance in health and disease. The human gut microbiome exhibits a high degree of plasticity, mainly in response to dietary changes that support a healthy gut ecosystem and minimize disease risk (146).The onset of new methodologies, including NGS and bioinformatic pipelines, have resulted in a paradigm shift in the fields of clinical microbiology and infectious diseases due to the realization of the complex interactions that occur within the microbiome. The relationship between human pathogens, infectious diseases, and the gut microbiome are slowly being revealed. Several studies have examined the correlation between the human gut microbiome and health status (141, 142). Reports have indicated that while the gut microbiome appears to be relatively stable under healthy conditions, any qualitative or quantitative changes in the gut microbiome can result in functional modifications and disease as reported (144, 147–149). A rich level of bacterial diversity is considered to be an indicator of a healthy status, while a low level of bacterial diversity is correlated with inflammatory, immune, and obesity-related diseases (58, 144, 147–153). Several studies have indicated that the human microbiota plays a crucial role in human health and disease (68, 154–168). Studies have also revealed that microbial symbiosis plays a central role in the development of a number of diseases, including liver diseases (156), metabolic disorders (154), gastrointestinal (GI) malignancy (157), respiratory diseases (158), autoimmune diseases (160), and mental or psychological diseases (160). Johnson et al. (169) discussed the *Bacteroidetes*, one of main components of the microbiome, their genetic variability and contrasting effect on metabolic diseases such as obesity and type II diabetes (169). Yiu et al. (170) proposed that body weight, metabolism, and diseases such as obesity are affected by the interplay between the immune system, metabolism and microbiome (170). In discussing chronic IBD, Frick and Wehkamp (171) outlined some of the available therapeutic interventions that can be used to alter mucosal immunity and the composition of the microbiome. While studying the molecular aspects of human gut-brain interactions, Lee et al. (172) demonstrated how the microbiota influences host physiology and neurodegenerative and neurological developmental diseases (172).

BIOINFORMATIC PIPELINES FOR METAGENOMIC DATA ANALYSIS

The advances in NGS have resulted in the production of massive datasets that are increasingly difficult to analyse (128). As larger datasets are generated, more sophisticated computational resources and bioinformatic tools are required. The interpretation and understanding of metagenomic studies depend on the computational tools that can be used to analyse enormous data sets and mine valuable, useful, and valid information regarding the microbial communities being studied. Bioinformatic tools used for metagenomic analysis, especially for translating raw sequences into meaningful data, are continually developing with the aim of providing the ability to examine both the taxonomic and the functional composition of diverse metagenomes (173, 174). A number of the specialized software programs available for analysing the metagenomic data are listed (**Table 2**). Based on the list provided, an example of a comparative analysis pipeline is presented in the present review that takes into consideration user friendliness, ease of access, open source availability, ability to analyse metagenomic datasets, and ability to provide graphical representations of the analyzed data (**Figure 5**). A description of the software (MG-RAST, EBI, QIIME and Mothur) used in the different pipelines is described in **Table 3**, which provides a detailed summary

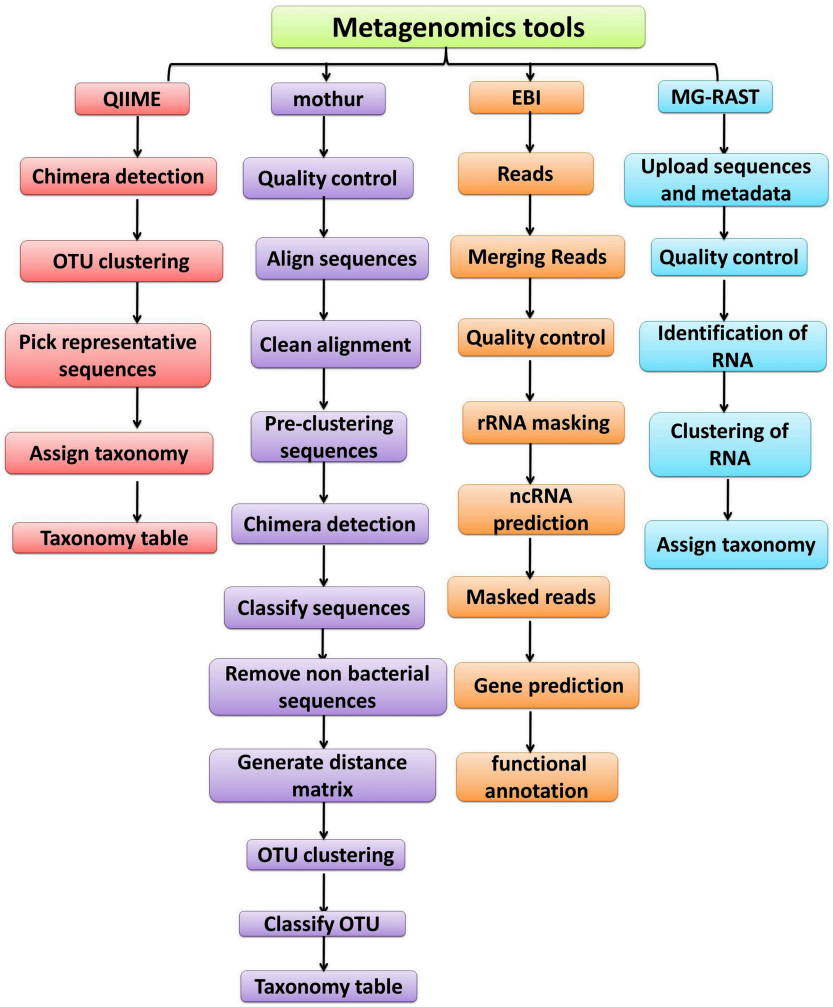


FIGURE 5 | Overview of the workflow used by metagenomic analysis tools (QIIME, Mothur, EBI and MG-RAST).

of the functionality and features of the mentioned software programs. The four pipelines share several steps during the analysis such as quality control, clustering, and annotation (**Figure 5**).

METAGENOMIC DATA ANALYSIS SOFTWARE: COMMAND BASED VS. GRAPHICAL USER INTERFACE

As comprehensive metagenomic studies are becoming more common, they are yielding novel and important insights into the microbial communities in diverse environments; from terrestrial to aquatic ecosystems and from human skin to the human gastrointestinal tract. Advances in NGS have made it more possible than ever for researchers to conduct whole genome sequencing. The analysis of the datasets obtained from NGS is complex and require an intelligent and systematic approach to process the data efficiently. The results obtained from any metagenomic study relies on *in silico* computational tools that can analyse large data sets and can mine and highlight various aspects about the community being examined. Although the tools and databases developed to investigate the taxonomic composition of a microbial community and provide information on the functional aspects of the community are becoming more elaborate and complex, though CLC microbial genomic package offered by Qiagen are good for these analysis. Nanopore sequencing technology has presented an option for an analysis pipeline, with novel options for assembly and annotation. **Figure 6**, presents the workflow involved in metagenomic analysis, and indicates all the steps and tools used for analyzing the data generated from metagenomic sequencing. The metagenomic pipeline can utilize any of the presented approaches, based on type of sequencing data (targeted metagenomics or shotgun metagenomics). The flowchart summarizes the basic steps that are followed in the analysis pipeline starting from preprocessing of the sequencing data to the final extraction, storage, and presentation of the data. The most popular tools, along with the databases and algorithms employed for the analysis, are indicated.

TABLE 3 | Comparative workflow of the four most commonly used bioinformatics pipeline for analyzing metagenomic datasets.

	EBI	MGRAST	QIIME/QIIME 2	MOTHUR
License	Free open-source	Free open-source	Free open-source	Free open-source
Implementation (release candidate)	Python	Python	Python	C++
Current Version available (March 2018)	4.1	4.0.3	1.9.1 and 2017.6.0, respectively	1.39.5
Website	http://www.ebi.ac.uk/metagenomics	http://metagenomics.anl.gov/	http://qiime.org/ and https://qiime2.org/	http://www.mothur.org/
Primary Usage	GUI	GUI	CL and GUI, respectively	CL
Amplicon data Analysis	Yes	Yes	Yes	Yes
Whole genome shotgun analysis	Yes	Yes	Yes but only experimental	No
Sequencing technology compatibility	Sanger, PacBio, Ion Torrent, Illumina, Nanopore	Sanger, PacBio, Ion Torrent, Illumina, Nanopore	Sanger, PacBio, Ion Torrent, Illumina, Nanopore	Sanger, PacBio, Ion Torrent, Illumina, Nanopore
Quality control	Yes	Yes	Yes	Yes
16S rRNA gene Databases searched	Silva, Rfam, MAPSeq, Pfam, TIGRFAM, Prints, Prosite patterns, Gene 3d	Silva, M5RNA, RDP and Greengenes	Greengenes, RDP, Siva and Unite	RDP, Greengenes Silva and Unite
Alignment method	PyNAST, MUSCLE, INFERNAL	BLAT	PyNAST, MUSCLE, INFERNAL	Needleman-Wunsch, Blastn, Gotoh
Taxonomic assignment	UCLUST, BLAST, Mothur, RDP	BLAT	UCLUST, BLAST, Mothur, RDP	Wang/RDP approach
Clustering algorithm	UCLUST, BLAST Mothur, CD-HIT	UCLUST	UCLUST, BLAST Mothur, CD-HIT	Mothur, CD-HIT and adapts DOTUR
Diversity analysis	Alpha and beta	Alpha	Alpha and beta	Alpha and beta
Phylogenetic Tree	YES	YES	FastTree	Clear cut algorithm
Visualization	T, BC, PC, HM, SC, PCA, Krona and Circos	T, BC, PC, HM, SC, PCA, Krona and Circos	T, BC, PC, HM, SC, PCA	T, BC, PC, HM, SC, PCA, Dendrograms, Venn diagrams
Submitted projects as on March 2018	Total: 1,653 Public: 1,503 Private:151	Total: 3,24,846 Public:52,615 Private:272,231	NA	NA

BC, Bar-Charts; BLAT, Blast like Alignment Tool; CL, Command Line; EBI, European Bioinformatics Institute; GUI, Graphical User Interface; HM, Heat Map; MGRAST, Metagenomic Rapid Annotations using Subsystems Technology; OUT, Operational Taxonomic Unit; PC, Pie-Charts; PCA, Principal Component Analysis; QIIME, Quantitative Insights into Microbial Ecology; RDP, Ribosomal Database Project; SC, Stacked Columns; T, Tabulation.

TECHNOLOGY AND THE CHANGING LANDSCAPE OF METAGENOMIC RESEARCH

Over the past decade, advancements in NGS have led to a significant reduction in the cost of genome sequencing. These technological advances have enabled the sequencing of several genomes in a day at a cost of approximately $1,000 per genome (**Figure 7**). The cost estimates presented in **Figure 7** represent (A) cost in U.S, dollars per Mb of sequence data from 2001 to 2009, (B) cost in U.S, dollars per Mb from 2009 to 2017, (C) cost in U.S, dollars per Genome from 2001 to 2009, and (D) cost in U.S, dollars per Genome from 2009 to 2017. Although sequencing is now relatively easy and straight forward, NGS technology is not perfect and errors in the data do occur. Moreover, some regions of the DNA have not been successfully sequenced. The underlying costs associated with different approaches to sequencing genomes are of great importance because they impact the scope and scale of genomic projects. Decreases in sequencing costs have led to the establishment of large collaborative projects with broad goals and individual laboratories targeting more specific questions.

The decreasing cost structure of DNA sequencing has had and will continue to have an impact on genomics and bio-computing. With the size of databases expanding continuously, the translation of data into biological insight is becoming more and more important. As a result, data analysis a more prominent aspect in obtaining information and value from the data (217). Significant analytical efforts are needed to gain useful insights from the generated data. The fields of microbiology, biotechnology, and medicine are already benefiting from genome sequencing efforts, and as costs continue to decrease, the practice of genome sequencing is expected to become almost routine. For example, the Sanger Institute is sequencing the genomes of patients suffering from cancer and rare diseases as part of the 100,000 Genomes Project organized by Genomics England.

Some patients have already benefitted from metagenomic-based diagnoses and treatments, and researchers are continuing to gain more knowledge about the genetic variations that cause a variety of diseases. Sequencing, however, is not the only option

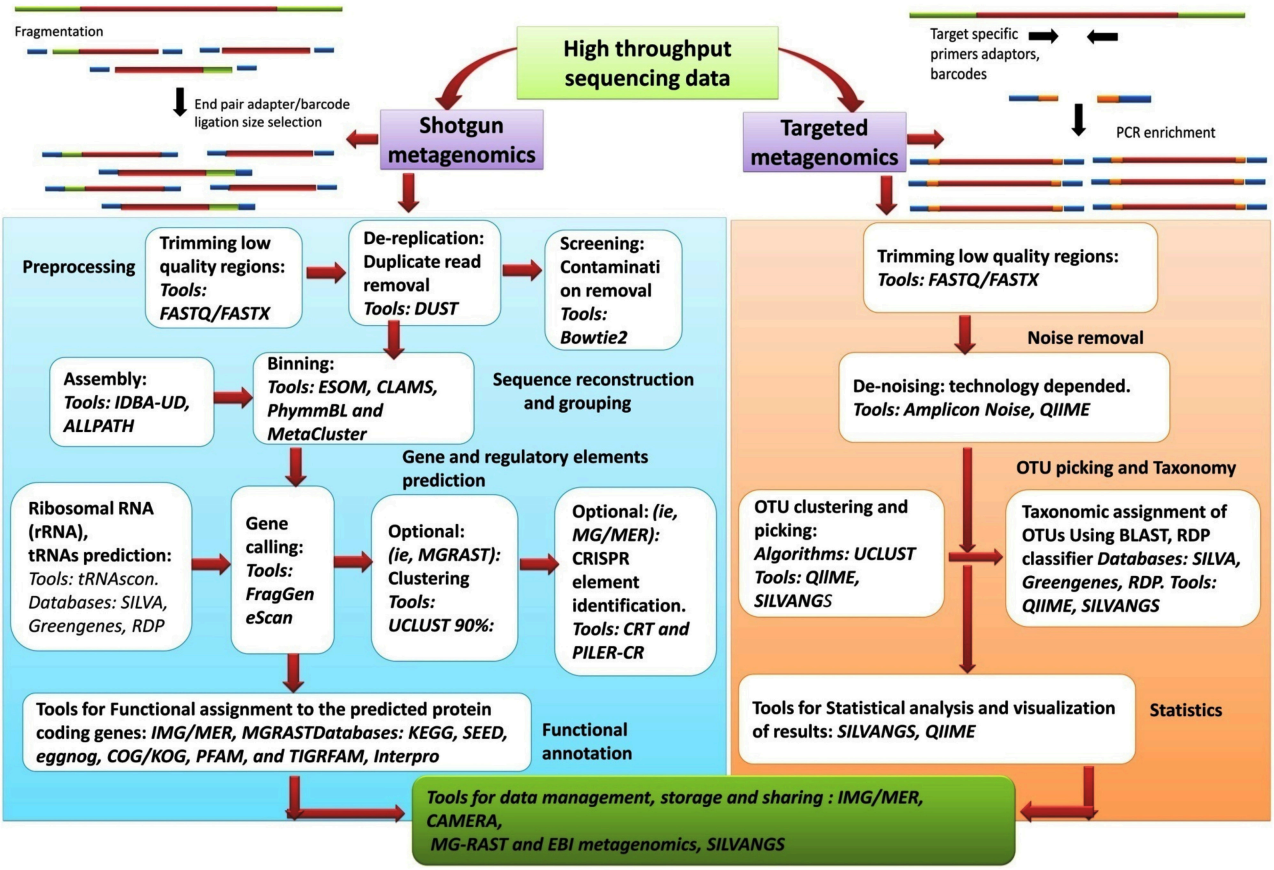


FIGURE 6 | Flow chart of basic metagenomics steps and tools currently in use.

for genetic analysis. An important part of the Precision Medicine Initiative, organized by the US National Institute of Health, is to develop a more predictable and possibly less technically complex method of genetic analysis. Sequencing, however, appears to be the only way to comprehensively explore the complex features of DNA that guide the initiation and progression of a number of diseases. Additionally, comprehensive sequencing also helps determine how our DNA keeps us healthy (218).

FUTURE PERSPECTIVES OF METAGENOMICS AND HUMAN HEALTH

Though the field of metagenomics pre-dates NGS, modern high-throughput sequencing technologies have greatly transformed this promising field by enabling a comprehensive characterization of all microorganisms present in a sample. As metagenomic approaches become more developed and clinically corroborated, it is expected that metagenomics will be at the forefront as a method for diagnosing infectious diseases. When a complex or unknown infectious disease is encountered, the use of multiple conventional diagnostic tests can potentially lead to unnecessary expenses; more importantly, this can also result in the delay of a diagnosis. Metagenomics can be used to identify potential pathogens, both known and novel, and can also be used to assess the state of an individual's microbiome. As sequencing become easier, faster, and more cost-effective, it will be possible to serially characterize the human microbiota to explore changes that occur in the human microbiome over time. This knowledge could lead to the development of novel medicines and approaches for treating infectious diseases. Indeed, metagenomic studies may become so standard that DNA sequencers could be used in homes to monitor changes in the stool microbiome of an individual to guide the maintenance of health.

CONCLUSIONS

All forms of life on this planet are dependent on microbes. They define an environment and are in turn defined by it. Our understanding of host-pathogen systems, however, is only in its infancy. Over the past two decades, sequencing technology, along with bioinformatic tools, have improved significantly; making it feasible to explore microbial communities residing within diverse hosts. There is a strong recognition that the microbial diversity existing in extreme habitats has largely been unexplored. To gain insight into this "latent" microbial flora, novel methodologies are required. NGS technologies have provided a rapid, cost-efficient

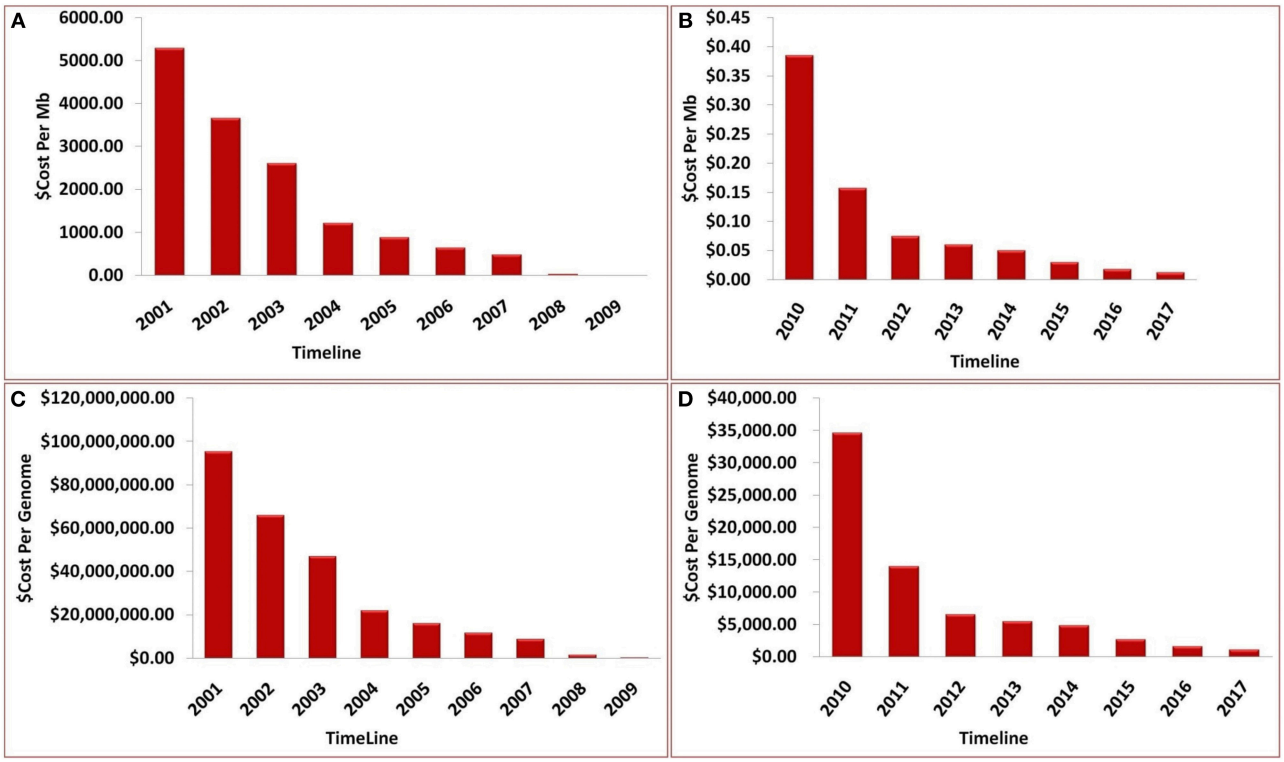


FIGURE 7 | Timeline showing the sequencing cost **(A)** per Mb until year 2009, **(B)** per Mb between year 2009 and 2017, **(C)** per genome until year 2009, **(D)** per genome between year 2009 and 2017.

means of generating sequencing data and provided sequencing platforms that can be used in large genome-sequencing centers, as well as individual laboratories. Illumina, PacBio, and Applied Biosystems, have all announced upgraded versions of their respective DNA-sequencing platforms. These upgrades will increase high-throughput ability and read length, while at the same time significantly reduce the cost of sequencing per base. These developments will significantly contribute to and provide exciting new opportunities to microbiologists. The integration of several approaches to biological studies will be necessary to answer questions about the diversity and ecology of microbial flora. It is the opinion of the authors of the present review that the development of better bioinformatic tools for analysing metagenomic data is urgently needed. The vast amounts of metagenomic data that will be forthcoming will bring new challenges for analysing, storing, and transferring data. Genome-sequencing centers and laboratories are going to become more dependent on information technology and bioinformatics. Bioinformatic expertise will increasingly be necessary to analyse large amounts of data and to mine the data for useful information about microbial diversity. Metagenomics will play an increasing role in the fields of medicine, biotechnology, and environmental science. The authors hope that this review provides a clear overview of the sequencing platforms and bioinformatic analysis of software that are available, including their high value and limitations.

AUTHOR CONTRIBUTIONS

MM prepared the draft of the manuscript under the guidance of AK and SY. AD prepared the illustrations. EA and AH edited the manuscript.

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Multidrug-Resistant *Pseudomonas aeruginosa* Accelerate Intestinal, Extra-Intestinal, and Systemic Inflammatory Responses in Human Microbiota-Associated Mice With Subacute Ileitis

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The globally rising incidences of multidrug-resistant (MDR) *Pseudomonas aeruginosa* (Psae) in humans and live-stock animals has prompted the World Health Organization to rate MDR Psae as serious threat for human health. Only little is known, however, regarding factors facilitating gastrointestinal Psae-acquisition by the vertebrate host and subsequently induced inflammatory sequelae. In the present study, we addressed whether subacute ileitis predisposed mice harboring a human gut microbiota for intestinal MDR Psae carriage and whether inflammatory responses might be induced following peroral challenge with the opportunistic pathogen. To accomplish this, secondary abiotic mice were associated with a human gut microbiota by fecal microbiota transplantation. Ten days later (i.e., on day 0), subacute ileitis was induced in human microbiota associated (hma) mice by peroral low-dose *Toxoplasma gondii* infection. On day 5 post-infection, mice were perorally challenged with 10^9 colony forming units of a clinical MDR Psae isolate by gavage and the fecal bacterial loads surveyed thereafter. Four days post-peroral challenge, only approximately one third of mice with a human gut microbiota and subacute ileitis harbored the opportunistic pathogen in the intestinal tract. Notably, the gut microbiota composition was virtually unaffected by the Psae-carriage status during subacute ileitis of hma mice. The Psae challenge resulted, however, in more pronounced intestinal epithelial apoptotic cell and T lymphocyte responses upon ileitis induction that were not restricted to the ileum, but also affected the large intestines. Higher Psae-induced abundances of T cells could additionally be observed in extra-intestinal compartments including liver, kidney, lung, and heart of hma mice with subacute ileitis. Furthermore, higher apoptotic cell numbers, but lower anti-inflammatory IL-10 concentrations were assessed in the liver of Psae as compared to mock treated mice with

ileitis. Remarkably, Psae-challenge was accompanied by even more pronounced systemic secretion of pro-inflammatory cytokines such as TNF and IL-6 at day 9 post ileitis induction. In conclusion, whereas in one third of hma mice with subacute ileitis Psae could be isolated from the intestines upon peroral challenge, the opportunistic pathogen was responsible for inflammatory sequelae in intestinal, extra-intestinal, and even systemic compartments and thus worsened subacute ileitis outcome irrespective of the Psae-carrier status.

Keywords: multi-drug resistant *Pseudomonas aeruginosa*, subacute ileitis, susceptibility to infection, host-pathogen-interaction, human microbiota associated mice

INTRODUCTION

Pseudomonas aeruginosa (Psae) constitute Gram-negative, non-fermenting bacteria that grow under strictly aerobic conditions and can be preferably found in moist environments (1). Bacterial motility and adhesive properties are provided by a single flagellum and several cell surface pili. A plethora of additional virulence factors such as alginate secretion, biofilm formation, quorum-sensing, an elaborate secretion system as well as intrinsic expression of antibiotic resistance genes facilitate adaptation to the respective environment as well as immune escape and establishment in the ecosystem of the vertebrate host (1, 2). Psae are considered opportunistic pathogens, but may cause urinary tract infections, ventilator-associated pneumonia, surgical and burn wound as well as bloodstream infections, particularly in hospitalized individuals with immunosuppressive comorbidities such as neutropenia that are associated with mortality rates of >30% (3). Furthermore, patients suffering from chronic respiratory morbidities including chronic obstructive lung disease, bronchiectasis, or cystic fibrosis are prone to chronic Psae infection, further exacerbating the underlying disease (3). Particularly the emergence of multidrug-resistant (MDR) Psae strains due to bacterial expression of resistance genes such as β-lactamases, 16S rRNA methylases, and carbapenemases in recent years leading to severe infections with high mortality rates due to very limited treatment options has prompted the World Health Organization (WHO) in 2017 to rate MDR Gram-negative bacteria including Psae as serious global threats to human health, further emphasizing the need for novel (antibiotics-independent) treatment strategies (4).

In the healthcare setting, contaminated devices including respiratory equipment, but also water bottles and sinks are typical external sources for Psae acquisition (5, 6). Whereas, Psae is not considered as part of the commensal gut microbiota, a previous report revealed that rectal opportunistic bacterial carriage was a predictor for Psae-associated infections in patients admitted to an intensive care unit (ICU) lateron (7). Thus, the human gastrointestinal tract (GIT) should be taken into consideration as a potential internal Psae reservoir. Particularly in cases, when the integrity of the complex gut microbiota is disturbed following antimicrobial therapy, for instance, the compromised colonization resistance [physiologically preventing the host from establishment of (opportunistic) pathogens (8, 9)] may facilitate stable Psae colonization within the human gastrointestinal ecosystem (10, 11). In an earlier report, Psae could be isolated from fecal samples of healthy volunteers up to 1 week following ingestion with decreasing loads over time, whereas the challenged individuals did not exhibit any clinical complications (12). Valid information regarding the immunopathological potential of intestinal Psae colonization in the vertebrate host is scarce, however.

Recently, our group provided evidence for the first time that intestinal carriage of a clinical MDR Psae isolate resulted in pronounced pro-inflammatory sequelae in intestinal as well as systemic compartments of healthy wildtype (WT) mice (13, 14). Furthermore, pre-existing inflammatory conditions in the gut facilitated stable intestinal Psae colonization in mice (15, 16), which subsequently aggravated chronic colitis (16). In the present study we addressed whether peroral challenge of mice suffering from more acute intestinal inflammatory conditions in a different anatomic compartment within the GIT (namely, subacute ileitis) worsened the outcome of the underlying disease. Since the host specific gut microbiota is known to be essentially involved in the onset, progress and outcome of distinct immunopathological conditions including intestinal inflammation (8, 9, 17, 18), we aimed at mimicking human gut microbiota conditions in the applied experimental model. We therefore generated mice that were harboring a human gut microbiota following human fecal microbiota transplantation (FMT) and induced subacute ileitis by peroral low-dose *Toxoplasma gondii* infection as described by us recently (19, 20). We here show that approximately one third of human microbiota associated (hma) mice with subacute ileitis harbored Psae in their intestines upon oral challenge. The opportunistic pathogen was, however, responsible for pro-inflammatory immune responses in intestinal, extra-intestinal and even systemic compartments and, thus, worsened subacute ileitis outcome irrespective of the Psae-carrier status.

Abbreviations: CFU, colony forming units; FMT, fecal microbiota transplantation; GIT, gastrointestinal tract; H&E, hematoxylin and eosin; hma, human microbiota associated; HPF, high power field; ICU, intensive care unit; MDR, multidrug-resistant; MLN, mesenteric lymph nodes; NO, nitric oxide; PBS, phosphate buffered saline; Psae, *Pseudomonas aeruginosa;* SPF, specific pathogen free; TLR, Toll-like receptor; Treg, regulatory T cells; WHO, World Health Organization; WT, wildtype

MATERIALS AND METHODS

Ethics Statement

Mouse experiments were performed in accordance of the European Guidelines for animal welfare (2010/63/EU) and had been approved by the commission for animal experiments headed by the "Landesamt für Gesundheit und Soziales" (LaGeSo, Berlin; registration numbers G0097/12 and G0039/15) before. Clinical conditions including body weight loss were assessed daily to assure animal welfare. Mice displaying over 20% weight loss were subjected to cervical dislocation in accordance with the guidelines of the local legal authorities.

Generation of Mice With a Human Gut Microbiota by Fecal Microbiota Transplantation

Female C57BL/6j mice were maintained under specific pathogen-free (SPF) conditions in the Forschungseinrichtungen für Experimentelle Medizin (FEM, Charité - University Medicine, Berlin, Germany). Microbiota depleted (i.e., secondary abiotic) mice were obtained following broad-spectrum antibiotic treatment as described earlier (8, 17). In brief, 8-week-old mice were treated with a quintuple antibiotic cocktail containing ampicillin plus sulbactam (1 g/L; Ratiopharm, Germany), vancomycin (500 mg/L; Cell Pharm, Germany), ciprofloxacin (200 mg/L; Bayer Vital, Germany), imipenem (250 mg/L; MSD, Germany), and metronidazole (1 g/L; Fresenius, Germany) via the drinking water for 8 weeks (*ad libitum*). Both, culture and 16S rRNA based molecular methods revealed virtual absence of bacteria in fecal samples as described previously (8, 18). Three days before human FMT the antibiotic cocktail was replaced by sterile tap water (*ad libitum*) to assure antibiotic washout (**Supplemental Figure S1**). Fresh fecal samples free of enteropathogenic bacteria, viruses, and parasites were collected from five individual healthy human volunteers, dissolved in sterile phosphate buffered saline (PBS; Gibco, Life Technologies, United Kingdom) and stored at −80°C as stated elsewhere (8). Individual fecal aliquots were thawed immediately before FMT and pooled. In turn, secondary abiotic mice were perorally challenged with the human fecal donor suspension on three consecutive days by gavage (8). Notably, the inter-experimental variations in bacterial loads of the donor suspensions were < 0.5 logarithmic orders of magnitude (**Supplementary Figure S2**). In order to guarantee proper establishment of the complex human gut microbiota within the murine host, mice were kept for 10 days before subacute ileitis induction. Immediately before peroral *T. gondii* infection (day 0) individual fecal samples were collected for quantitative molecular analyses of main intestinal bacterial communities as described elsewhere (8, 17, 21).

Molecular Analysis of the Human Fecal Donor Suspensions and the Intestinal Microbiota

Fresh ileal and colonic luminal samples were immediately snap-frozen in liquid nitrogen and stored at −80°C until further processing. DNA was extracted from fecal samples as reported earlier (17). In brief, for quantification of DNA the Quant-iT PicoGreen reagent (Invitrogen, UK) was applied and DNA in samples adjusted to 1 ng per μL. Then, total eubacterial loads as well as the main bacterial groups within the human gut microbiota such as enterobacteria, enterococci, lactobacilli, bifidobacteria, *Bacteroides/Prevotella* species, *Clostridium coccoides* group, and *Clostridium leptum* group were surveyed by quantitative real-time polymerase chain reaction using genera-, group- or species-specific 16S rRNA gene primers (Tib MolBiol, Germany) as stated elsewhere (21, 22) and numbers of 16S rRNA gene copies per ng DNA of each sample assessed.

Induction of Subacute Ileitis

On day 0, mice were infected perorally with one cyst of *T. gondii* ME49 strain (in a volume of 0.3 mL brain suspension) by gavage in order to induce subacute ileitis (**Supplemental Figure S1**), as reported recently (19).

MDR *P. aeruginosa* Infection and Quantitative Assessment of Fecal Bacterial Loads

The MDR Psae isolate was initially isolated from respiratory material of a patient with nosocomial pneumonia and kindly provided by Prof. Dr. Bastian Opitz (Charité–University Medicine, Berlin, Germany). Notably, the bacterial strain displayed antimicrobial sensitivity to fosfomycin and colistin only (15). Prior infection, the Psae strain was grown on cetrimide agar (Oxoid) for 48 h in an aerobic atmosphere at 37°C.

On day 5 post ileitis induction, mice were perorally challenged with 10^9 colony forming units (CFU) of the MDR Psae strain by gavage in a total volume of 0.3 mL PBS as reported earlier (**Supplemental Figure S1**) (15).

For quantitative assessment of Psae colonization densities in the intestinal tract post-challenge, fecal and intestinal luminal samples were homogenized in sterile PBS. Serial dilutions were then streaked onto Columbia agar supplemented with 5% sheep blood (Oxoid, Germany) and onto cetrimide agar and incubated in an aerobic atmosphere at 37°C for 48 h as described previously (15). Difference of the sample weights before and after asservation revealed respective fecal weights. The detection limit of cultivable bacteria was ∼100 CFU per g.

Cultural Survey of Bacterial Translocation

In order to survey viable bacteria translocating from the intestinal tract to extra-intestinal and systemic compartments, *ex vivo* biopsies derived from mesenteric lymph nodes (MLN), spleen, liver, kidney and lung were homogenized in sterile PBS and analyzed in serial dilutions on respective solid media as described earlier (17, 23, 24). Cardiac blood was incubated in thioglycolate enrichment broths (BD Bioscience, Germany) for 1 week at 37°C and streaked onto solid media thereafter (15). Bacteria were grown at 37°C for at least two and t3 days under aerobic, microaerobic and anaerobic conditions.

Clinical Conditions

Macroscopic and / or microscopic occurrence of blood in murine fecal pellets was surveyed in individual mice on a daily basis by the Guajac method (Haemoccult; Beckman Coulter/ PCD, Germany) as stated elsewhere (25).

Sampling Procedures

96 h following Psae challenge (i.e., day 9 post ileitis induction), mice were sacrificed by isoflurane treatment [Abbott, Germany (**Figure S1**)]. Cardiac blood (for serum) and tissue samples from spleen, liver, lung, heart, MLN, ileum, and colon were removed under sterile conditions. Respective *ex vivo* biopsies were collected from each mouse in parallel for microbiological, immunological and immunohistochemical analyses.

Histopathology

Ex vivo biopsies taken from the terminal ileum were fixed in 5% formalin and embedded in paraffin. Sections (5 µm) were stained with hematoxylin and eosin (H&E) and subjected to a standardized histopathological scoring system ranging from 0 to 6 as described in more detail earlier (17).

Immunohistochemistry

Five µm thin paraffin sections of ileal and colonic *ex vivo* biopsies were used for *in situ* immunohistochemical analysis as reported previously (21, 26–28). In brief, to assess apoptotic cells, proliferating cells, T lymphocytes and regulatory T cells (Treg), primary antibodies against cleaved caspase-3 (Asp175, #9661, Cell Signaling, Leiden, Netherlands; 1:200), Ki67 (clone 16A8, #652401, BioLegend/Biozol, Eching, Germany; 1:200), CD3 (#IR50361-2, Dako, Santa Clara, CA, USA; 1:5) and FOXP3 (clone FJK-165, #14-5773, eBioscience, Frankfurt, Germany; 1:100), respectively, were used. An independent blinded investigator determined the average numbers of positively stained cells in at least six high power fields (HPF, 0.287 mm^2; 400 x magnification).

Cytokine Detection

Ex vivo biopsies (∼1 cm^2) derived from colon and ileum (both cut longitudinally and washed in PBS), as well as from liver (∼1 cm^2), kidney (cut longitudinally, one half), lung (one organ), spleen (one half), and MLN (3 single lymph nodes) were transferred to 24-flat-bottom well culture plates (Falcon, Germany) containing 500 µL serum-free RPMI 1640 medium (Gibco, life technologies) supplemented with penicillin (100 U/mL, Biochrom, Germany) and streptomycin (100 µg/mL; Biochrom). After 18 h at 37°C, IFN-γ, TNF, MCP-1, IL-6, and IL-10 concentrations were measured in culture supernatants and serum by the Mouse Inflammation Cytometric Bead Assay (CBA; BD Bioscience) applying a BD FACSCanto II flow cytometer (BD Bioscience). Nitric oxide (NO) concentrations were determined by the Griess reaction as described earlier (17).

Statistical Analysis

Medians and levels of significance were determined by the one-way ANOVA test followed by Tukey post-correction test for multiple comparisons. Two-sided probability (p) values ≤ 0.05 were considered significant. Experiments were reproduced three times.

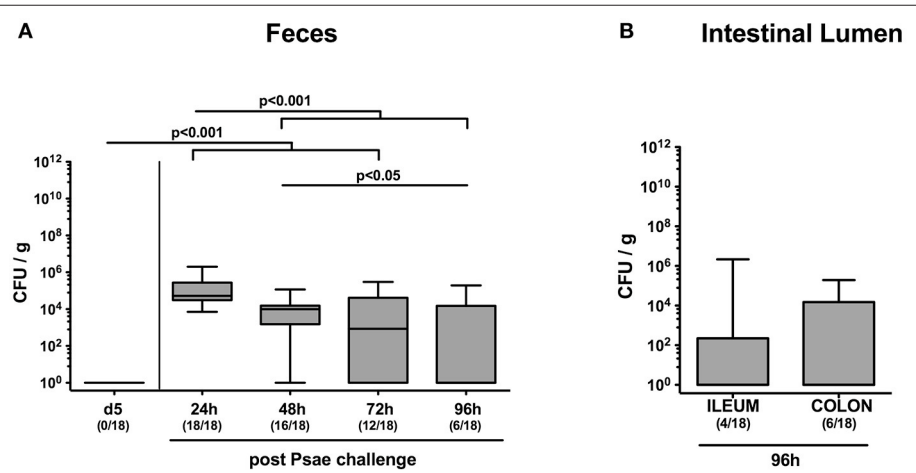

FIGURE 1 | Intestinal MDR *Pseudomoas aeruginosa* loads following peroral challenge of mice with a human gut microbiota suffering from subacute ileitis. Secondary abiotic mice were generated by broad-spectrum antibiotic treatment and associated with a complex human gut microbiota by fecal microbiota transplantation. Ten days thereafter, subacute ileitis was induced by peroral infection of human microbiota associated mice with one cyst of *T. gondii* ME49 strain (day 0). On day 5 post ileitis induction (p.i.), mice were perorally challenged with MDR *P. aeruginosa* (Psae) and **(A)** the fecal Psae loads were followed up for further 96 h by culture. **(B)** In addition, Psae counts were assessed in the small as well as large intestinal lumen 96 h post Psae challenge. Box plots represent the 75th and 25th percentiles of the median (black bar inside the boxes). Total range and significance levels determined by one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are shown. Numbers of mice harboring Psae in the respective intestinal compartment out of the total numbers of analyzed animals are given in parentheses. Data were pooled from four independent experiments.

RESULTS

Colonization Properties of MDR *P. aeruginosa* in the Intestinal Tract of Mice With a Human Gut Microbiota Suffering From Subacute Ileitis

In the present study we raised the question whether subacute ileitis predisposed mice with a human gut microbiota for intestinal MDR Psae carriage and whether intestinal, extra-intestinal or even systemic inflammatory sequelae might be induced following peroral Psae challenge. To address this, secondary abiotic mice were associated with a complex human gut microbiota by FMT on three consecutive days. Ten days later (i.e., on day 0), subacute ileitis was induced by peroral low-dose *T. gondii* infection of hma mice. On day 5 post ileitis induction, mice were perorally challenged with 10^9 CFU of a clinical MDR *P. aeruginosa* isolate by gavage and the fecal opportunistic pathogenic loads surveyed thereafter (**Supplemental Figure S1**). As early as 24 h following peroral challenge, fecal Psae could be cultured from all mice with median loads of $\sim 10^5$ CFU per g (**Figure 1A**). At later time points (48, 72, and 96 h, respectively), however, both fecal Psae positivity rates (88.9, 66.7, and 33.3%, respectively) as well as median Psae loads ($p < 0.001$; **Figure 1A**) were lower, whereas at the end of the experiment, Psae could be isolated from the ileal and colonic lumen in 22.2% and 33.3% of cases, respectively, (**Figure 1B**). Hence, only one third of mice with a human gut microbiota and subacute ileitis harbored MDR Psae in their intestinal tract 4 days post peroral challenge.

Gut Microbiota Changes in Mice With a Human Gut Microbiota and Subacute Ileitis Following MDR *P. aeruginosa* Challenge

We next addressed whether MDR Psae challenge following subacute ileitis induction resulted in intestinal microbiota changes in mice with a human gut microbiota (**Figures 2A–H**). Quantitative culture-independent analysis revealed that until day 9 following ileitis induction mock treated hma mice displayed increased ileal loads of enterobacteria and enterococci ($p < 0.05$; **Figures 2B,C**), whereas both ileal *Clostridium coccoides*

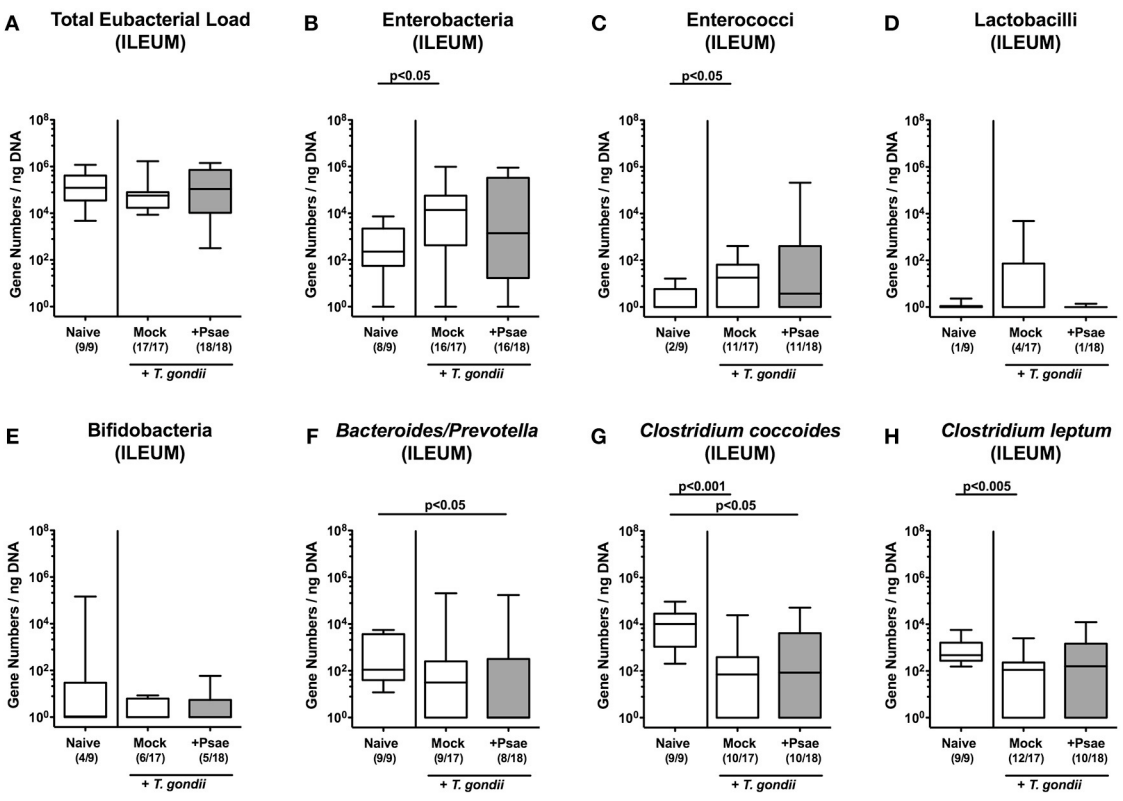

FIGURE 2 | Ileal microbiota changes following MDR *P. aeruginosa* challenge of mice with a human gut microbiota suffering from subacute ileitis. Subacute ileitis was induced in mice harboring a human gut microbiota by peroral *T. gondii* infection on day (d) 0. At day 5 post ileitis induction mice were either perorally challenged with MDR *P. aeruginosa* (+Psae, dissolved in PBS) or with PBS alone (Mock). Uninfected mice with a human gut microbiota but without ileitis served as control animals (Naive). **(A–H)** The microbiota composition of the ileal lumen was determined 96 h later by quantitative Real-Time PCR amplifying bacterial 16S rRNA variable regions of the main intestinal bacterial groups (expressed as 16S rRNA gene numbers per ng DNA) including the total eubacterial load. Box plots represent the 75th and 25th percentiles of the median (black bar inside the boxes). Total range and significance levels determined by one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are shown. Numbers of mice harboring respective bacterial groups in the ileal lumen out of the total numbers of analyzed animals are given in parentheses. Data were pooled from four independent experiments.

and *Clostridium leptum* group gene numbers were lower as compared to naive hma mice without ileitis ($p < 0.001$ and $p < 0.005$, respectively; **Figures 2G,H**). At day 9 post ileitis induction, lower *Bacteroides / Prevotella* species and *Clostridium coccoides* group loads could be detected in the ileal lumen of Psae challenged as compared to naive mice ($p < 0.05$; **Figures 2F,G**).

We additionally assessed the microbiota composition in the large intestinal tract (**Figures 3A–H**). Within 9 days following ileitis induction, total eubacterial loads increased in the colonic lumen of both mock and Psae challenged mice ($p < 0.01$ and $p < 0.005$, respectively; **Figure 3A**). This also held true for large intestinal enterobacteria ($p < 0.005$ and $p < 0.001$, respectively; **Figure 3B**) and enterococci ($p < 0.01$; **Figure 3C**), whereas Psae challenged animals displayed lower lactobacilli loads in their colon as compared to mock treated controls at day 9 p.i. ($p < 0.05$; **Figure 3D**). Hence, the intestinal loads of assessed bacterial taxa, groups and species did not differ between Psae challenged and unchallenged hma mice during subacute ileitis.

Macroscopic and Microscopic Inflammatory Sequelae of MDR *P. aeruginosa* Challenge in Mice With a Human Gut Microbiota Suffering From Subacute Ileitis

We next assessed whether peroral Psae challenge worsened subacute ileitis of hma mice. Mice of either treatment group displayed mean body weight loss of ∼5% during the observation period (not shown). At day 9 p.i., however, 58.8% of mock treated mice exhibited blood in their feces, whereas this was the case in 72.2% of Psae challenged and none of naive mice (**Figure 4A**). Following ileitis induction, mice of either cohort displayed comparable histopathological changes within the small intestinal mucosa and lamina propria ($p < 0.005$ vs. naive controls; **Figure 4B**). Of note, the range of histopathological scores was higher following Psae as compared to mock challenged mice at day 9 p.i., with single mice displaying even ileal necrosis in the former cohort, which was not the case in the latter group (**Figure 4B**).

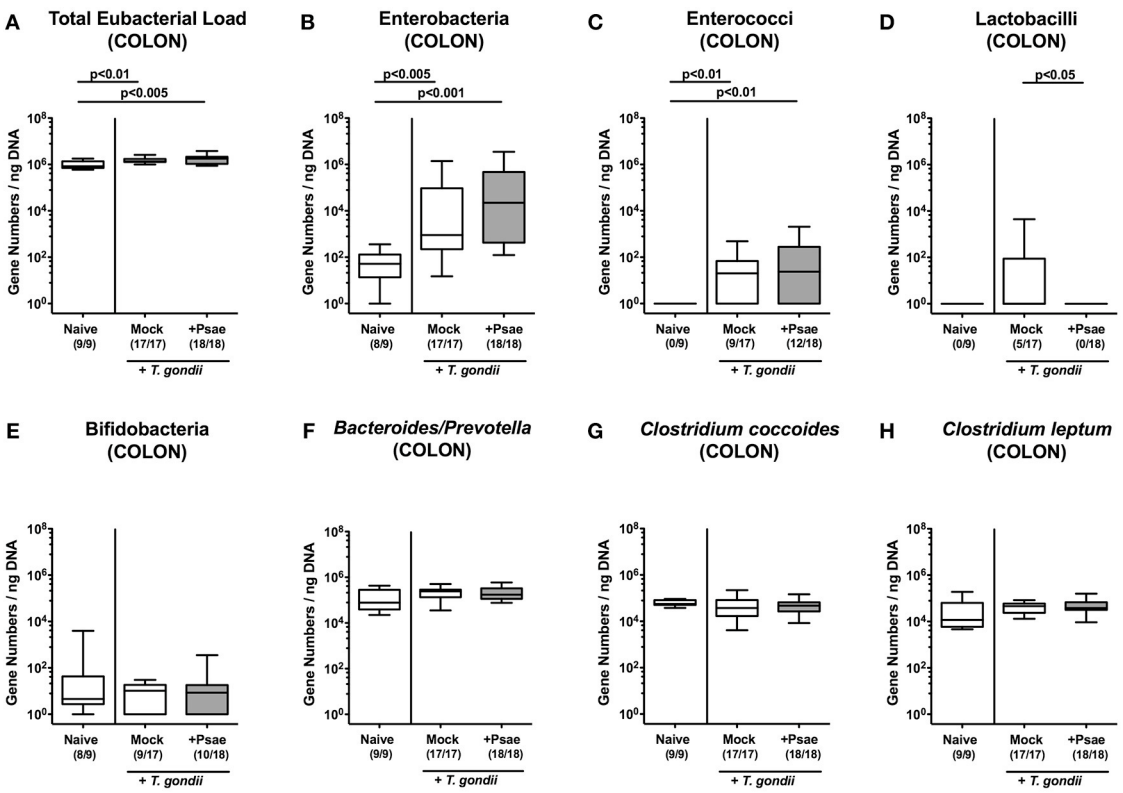

FIGURE 3 | Colonic microbiota changes following MDR *P. aeruginosa* challenge of mice with a human gut microbiota suffering from subacute ileitis. Subacute ileitis was induced in mice harboring a human gut microbiota by peroral *T. gondii* infection on day (d) 0. At day 5 post ileitis induction mice were either perorally challenged with MDR *P. aeruginosa* (+Psae, dissolved in PBS) or with PBS alone (Mock). Uninfected mice with a human gut microbiota but without ileitis served as control animals (Naive). **(A–H)** The microbiota composition of the colonic lumen was determined 96 h later by quantitative Real-Time PCR amplifying bacterial 16S rRNA variable regions of the main intestinal bacterial groups (expressed as 16S rRNA gene numbers per ng DNA) including the total eubacterial load. Box plots represent the 75th and 25th percentiles of the median (black bar inside the boxes). Total range and significance levels determined by one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are shown. Numbers of mice harboring respective bacterial groups in the colonic lumen out of the total numbers of analyzed animals are given in parentheses. Data were pooled from four independent experiments.

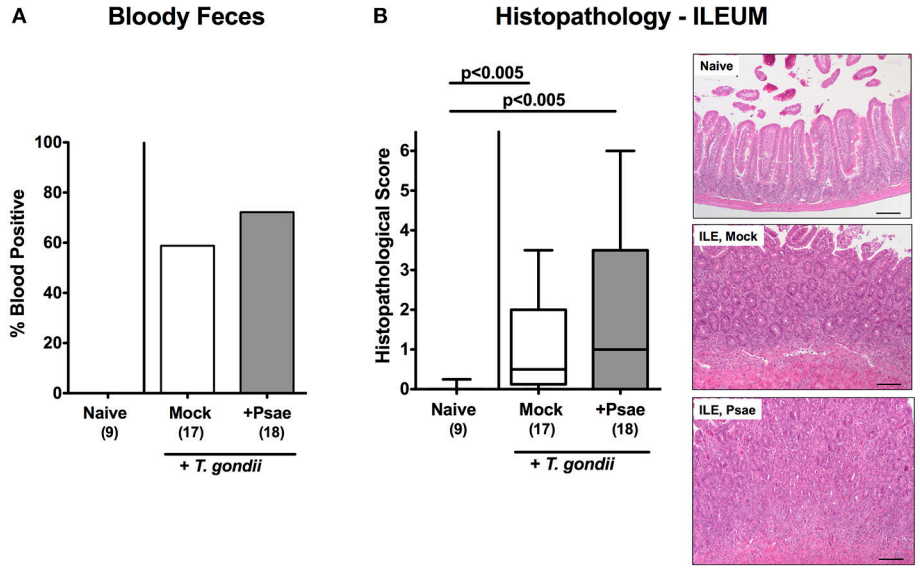

FIGURE 4 | Clinical and histopathological sequelae following peroral MDR *P. aeruginosa* challenge of mice with a human gut microbiota suffering from subacute ileitis. At day 5 following subacute ileitis induction, mice with a human gut microbiota were either perorally challenged with MDR *P. aeruginosa* (+Psae, dissolved in PBS) or with PBS alone (Mock). Uninfected mice with a human gut microbiota but without ileitis served as control animals (Naive). Clinical and microscopic intestinal changes were assessed 96 h following Psae challenge. **(A)** Abundance of blood was determined in fecal samples by the Guajac (Haemoccult) method. Cumulative blood positivity rates were calculated from four independent experiments. **(B)** Histopathological changes were determined in H&E stained ileal paraffin sections applying a standardized scoring system (see methods) and representative photomicrographs depicted (right panel; 100x magnification, scale bar 100 μm). Box plots represent the 75th and 25th percentiles of the median (black bar inside the boxes). Total range and significance levels determined by one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are shown. The total numbers of analyzed animals are given in parentheses. Data were pooled from four independent experiments.

Intestinal Apoptotic, Proliferating, and Immune Cell Responses Upon MDR *P. aeruginosa* Challenge of Mice With a Human Gut Microbiota Suffering From Subacute Ileitis

We further expanded our histopathological analyses and quantitatively assessed apoptotic cells in the small intestinal epithelia applying *in situ* imunohistochemistry. Ileitis induction was accompanied by increased numbers of caspase3+ ileal epithelial cells ($p < 0.001$; **Figure 5A**; **Supplemental Figure S3A**). Of note, at day 9 p.i., Psae challenged mice were suffering from even more distinct ileal epithelial apoptosis as compared to mock treated mice ($p < 0.01$; **Figure 5A**; **Supplemental Figure S3A**).

We further quantitated Ki67+ cells in ileal epithelia indicative for proliferative/regenerative cell responses counteracting potential Psae-induced cell damage. Until day 9 p.i., Ki67+ cell numbers had markedly increased in the ileal epithelia as compared to naive controls ($p < 0.001$; **Figure 5B**; **Supplemental Figure S3B**), but irrespective whether mice were Psae or mock challenged (n.s.; **Figure 5B**; **Supplemental Figure S3B**).

Given that *T. gondii* induced ileitis comprises a T cell-driven immunopathological scenario (29), we next assessed T lymphocyte counts in ileal paraffin sections by *in situ* imunohistochemistry. At day 9 p.i., *T. gondii* infected mice displayed increased numbers of CD3+ cells in their ileal mucosa and lamina propria, but exhibited even higher T cell counts upon Psae as compared to mock challenge ($p < 0.001$; **Figure 5C**; **Supplemental Figure S3C**). Interestingly, increases in small intestinal T cell numbers following ileitis induction were paralleled by elevated numbers of FOXP3+ cells in the ileal mucosa and lamina propria of both, Psae and mock challenged mice at day 9 p.i. ($p < 0.001$; **Figure 5D**; **Supplemental Figure S3D**) with higher counts in the former as compared to the latter ($p < 0.001$; **Figure 5D**; **Supplemental Figure S3D**).

Even though peroral *T. gondii* infection of susceptible mice is well-known to affect primarily the terminal ileum (29), we additionally included the large intestines into our immunohistopathological survey. Interestingly, ileitis induction was also accompanied by increases in apoptotic cell numbers in colonic epithelia ($p < 0.001$; **Figure 5E**; **Supplemental Figure S3E**) with a trend toward even higher counts in Psae as compared to mock treated mice at day 9 p.i. (n.s. due to high standard deviations). Like in the ileum, *T. gondii* infection resulted in increased Ki67+ colonic epithelial numbers ($p < 0.001$; **Figure 5F**; **Supplemental Figure S3F**), that were even more pronounced in Psae as compared to mock challenged mice ($p < 0.01$; **Figure 5F**; **Supplemental Figure S3F**). Furthermore, *T. gondii* induced elevated numbers of both CD3+ and FOXP3+ cells could be determined in the mucosa and lamina propria of the large intestines at day 9 p.i. ($p < 0.001$;

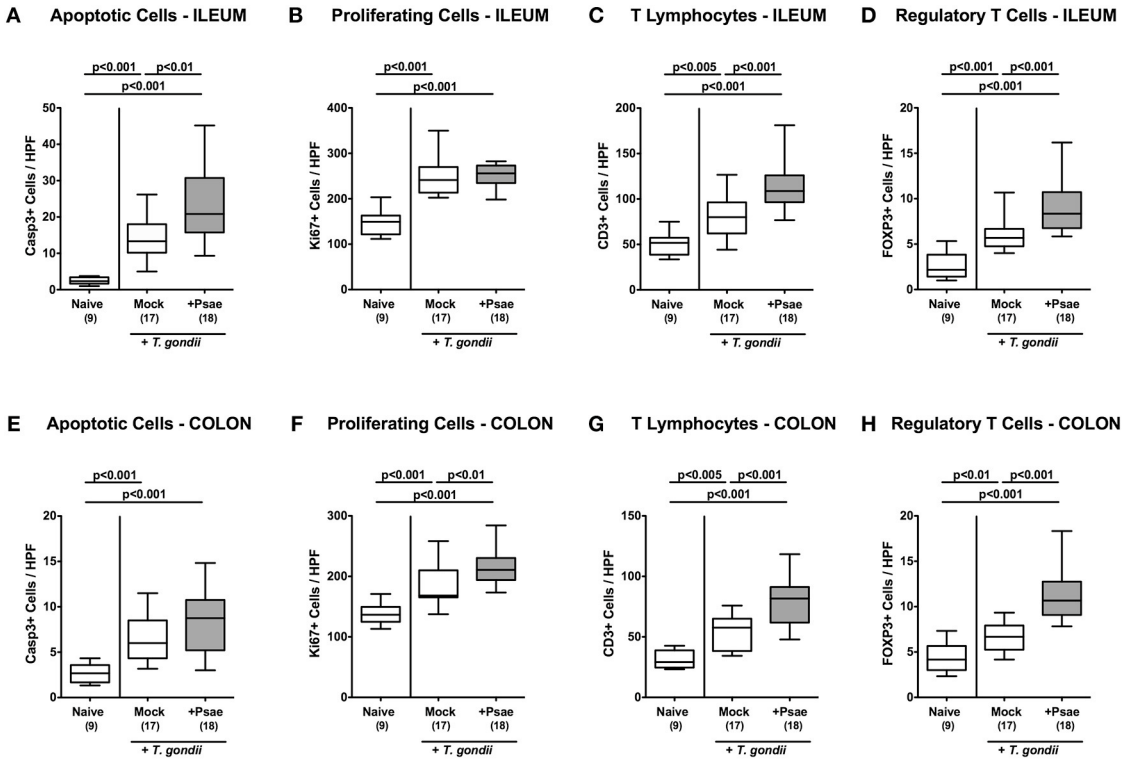

FIGURE 5 | Apoptotic and proliferating epithelial cell as well as immune cell responses in the ileum and colon following peroral MDR *P. aeruginosa* challenge of mice with a human gut microbiota suffering from subacute ileitis. At day 5 following subacute ileitis induction, mice with a human gut microbiota were either perorally challenged with MDR *P. aeruginosa* (+Psae, dissolved in PBS) or with PBS alone (Mock). Uninfected mice with a human gut microbiota but without ileitis served as control animals (Naive). 96 h following Psae challenge, the average numbers of apoptotic (Casp3+; **A**,**E**) and proliferating (Ki67+; **B**,**F**) epithelial cells as well as of T lymphocytes (CD3+; **C,G**), and regulatory T cells (FOXP3+; **D,H**) in at least six high power fields (HPF) were quantitatively assessed in ileal **(A–D)** and colonic **(E–H)** paraffin sections applying *in situ* immunhistochemistry. Box plots represent the 75th and 25th percentiles of the median (black bar inside the boxes). The total range and significance levels determined by one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are shown. The total numbers of analyzed animals are given in parentheses. Data were pooled from four independent experiments.

Figures 5G,H; **Supplemental Figures S3G,H**) with even higher counts in the Psae vs. mock cohort ($p < 0.001$; **Figures 5G,H**; **Supplemental Figures S3G,H**). Hence, Psae challenge resulted in more pronounced intestinal epithelial apoptotic and T cell responses upon ileitis induction that were not restricted to the ileum, but also involved the large intestines.

Intestinal Pro-Inflammatory Mediator Responses Upon MDR *P. aeruginosa* Challenge of Mice With a Human Gut Microbiota Suffering From Subacute Ileitis

We next addressed whether changes in apoptotic and immune cell responses upon Psae challenge of hma mice with subacute ileitis was accompanied by pro-inflammatory mediator responses in the intestinal tract. Irrespective whether Psae or mock challenged, hma mice displayed increased IFN-γ concentrations in *ex vivo* biopsies derived from the ileum, colon or MLN at day 9 p.i. ($p < 0.001$ vs. naive controls; **Supplemental Figures S4A–C**). In addition, TNF and NO were multi-fold elevated in MLN following ileitis induction in either cohort ($p < 0.005$–0.001 vs. naive; **Supplemental Figures S4D,E**). Hence, Psae challenge did not result in further augmented pro-inflammatory mediator responses in the intestinal tract following subacute ileitis induction of hma mice.

Extra-Intestinal Apoptotic, Proliferating, and Immune Cell Responses Upon MDR *P. aeruginosa* Challenge of Mice With a Human Gut Microbiota Suffering From Subacute Ileitis

We next addressed whether peroral MDR Psae challenge of hma mice with subacute ileitis might lead to more pronounced inflammatory responses in extra-intestinal compartments including liver, kidney, lung, and heart. Nine days following ileitis induction, hma mice exhibited increased numbers of apoptotic hepatic cells ($p < 0.001$ vs. naive; **Figure 6A**; **Supplemental Figure S5A**) that were even higher upon Psae as compared to mock challenge ($p < 0.001$; **Figure 6A**; **Supplemental Figure S5A**). Increases in apoptotic cells were paralleled by elevated T lymphocyte and Treg counts in the livers of both, Psae and mock challenged mice at day 9

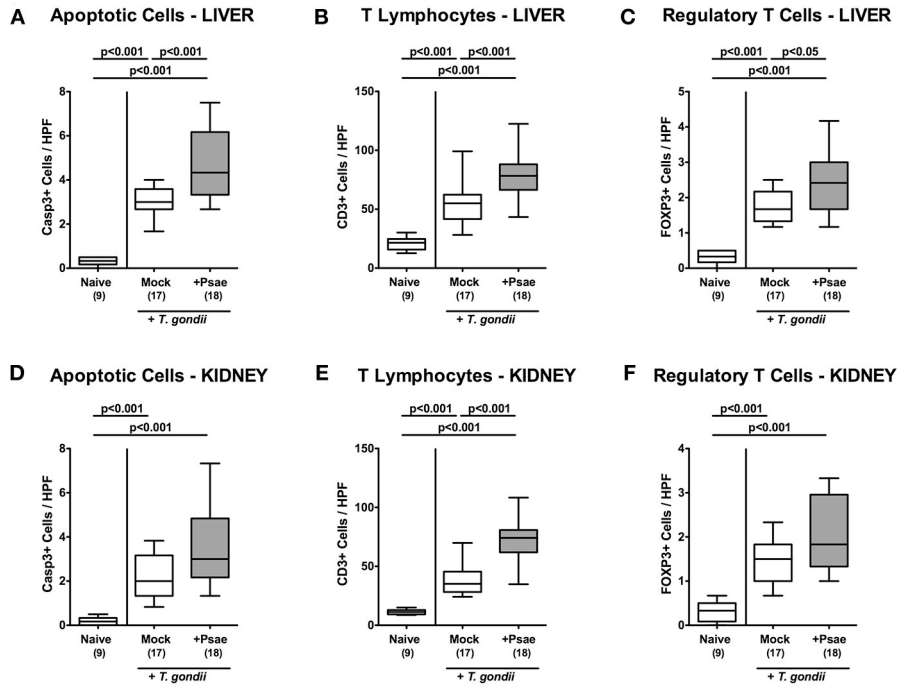

FIGURE 6 | Apoptotic epithelial cell as well as immune cell responses in the liver and kidney following peroral MDR *P. aeruginosa* challenge of mice with a human gut microbiota suffering from subacute ileitis. At day 5 following subacute ileitis induction, mice with a human gut microbiota were either perorally challenged with MDR *P. aeruginosa* (+Psae, dissolved in PBS) or with PBS alone (Mock). Uninfected mice with a human gut microbiota but without ileitis served as control animals (Naive). 96 h following Psae challenge, the average numbers of apoptotic (Casp3+; **A,D**) epithelial cells as well as of T lymphocytes (CD3+; **B,E**) and regulatory T cells (FOXP3+; **C,F**) in at least six high power fields (HPF) were quantitatively assessed in hepatic **(A–C)** and renal **(D–F)** paraffin sections applying *in situ* immunhistochemistry. Box plots represent the 75th and 25th percentiles of the median (black bar inside the boxes). The total range and significance levels determined by one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are shown. The total numbers of analyzed animals are given in parentheses. Data were pooled from four independent experiments.

p.i. ($p < 0.001$; **Figures 6B,C**; **Supplemental Figures S5B,C** with higher numbers in the former as compared to the latter ($p < 0.001$ and $p < 0.05$, respectively; **Figures 6B,C**; **Supplemental Figures S5B,C**.

In the kidney, apoptotic cells, CD3+ and FOXP3+ cells increased upon ileitis induction of hma mice until day 9 p.i. (**Figures 6D–F**, **Supplemental Figures S5D–F**). Additional Psae challenge resulted in more pronounced increases in renal T lymphocytes as compared to the mock cohort (**Figure 6E**; **Supplemental Figure S5E**), whereas a trend toward higher Casp3+ and FOXP3+ cell counts could be observed in the kidneys following Psae challenge at day 9 p.i. (n.s.; **Figures 6D,F**, **Supplemental Figures S5D,F**).

Also in lung and heart, elevated apoptotic cell numbers could be observed 9 days following ileitis induction in either cohort ($p < 0.005$ and $p < 0.05$ vs. naive, respectively; **Figures 7A,D**, **Supplemental Figures S6A,D**) that were accompanied by multi-fold increased T lymphocyte counts in both organs ($p < 0.001$; **Figures 7B,E**, **Supplemental Figures S6B,E**). Remarkably, increases in T cell numbers in lung and heart were more pronounced in Psae as compared to mock challenged mice ($p < 0.01$ and $p < 0.05$, respectively; **Figures 7B,E**, **Supplemental Figures S6B,E**). Only in the lung, but not in the heart, higher Treg numbers could be assessed at day 9 p.i. ($p < 0.001$; **Figures 7C,F**, **Supplemental Figures S6C,F**) with similar counts in either cohort. Hence, additional peroral Psae challenge resulted in increased apoptotic and T cell responses in extra-intestinal compartments.

Extra-Intestinal Inflammatory Mediator Responses Upon MDR *P. aeruginosa* Challenge of Mice With a Human Gut Microbiota Suffering From Subacute Ileitis

We further assessed whether the observed increased apoptotic and immune cell responses in extra-intestinal compartments upon Psae challenge of hma mice with subacute ileitis were accompanied by pronounced inflammatory mediator secretion. Nine days following ileitis induction increased concentrations of pro-inflammatory cytokines such as IFN-γ and TNF could be observed in the liver of hma mice ($p < 0.005$–0.001; **Figures 8A,B**), but with no differences between mice of either cohort. Interestingly, hepatic concentrations of the anti-inflammatory cytokine IL-10 were elevated in mock treated, but not Psae challenged mice at day 9 p.i. ($p < 0.005$ vs. naive, $p < 0.05$ vs. Psae; **Figure 8C**).

In the kidney, pro-inflammatory cytokines including IFN-γ, TNF, MCP-1, and IL-6 were increased to similar extent in Psae

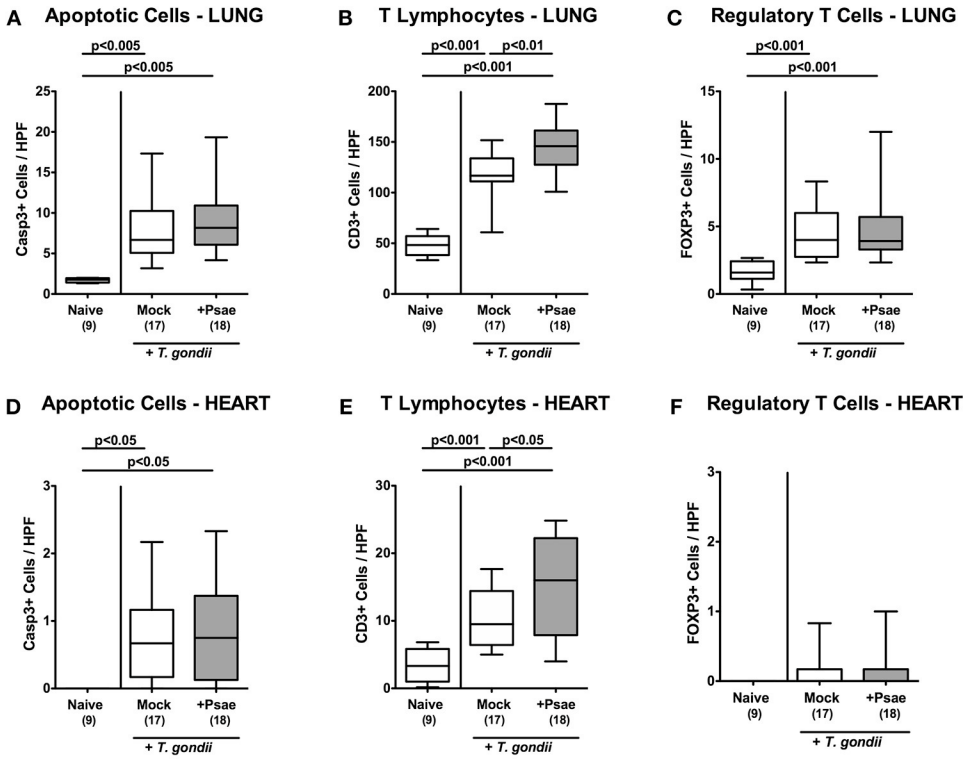

FIGURE 7 | Apoptotic epithelial cell as well as immune cell responses in the lung and heart following peroral MDR *P. aeruginosa* challenge of mice with a human gut microbiota suffering from subacute ileitis. At day 5 following subacute ileitis induction, mice with a human gut microbiota were either perorally challenged with MDR *P. aeruginosa* (+Psae, dissolved in PBS) or with PBS alone (Mock). Uninfected mice with a human gut microbiota but without ileitis served as control animals (Naive). 96 h following Psae challenge, the average numbers of apoptotic (Casp3+; **A,D**) epithelial cells as well as of T lymphocytes (CD3+; **B,E**) and regulatory T cells (FOXP3+; **C,F**) in at least six high power fields (HPF) were quantitatively assessed in pulmonal **(A–C)** and cardiac **(D–F)** paraffin sections applying *in situ* immunhistochemistry. Box plots represent the 75th and 25th percentiles of the median (black bar inside the boxes). The total range and significance levels determined by one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are shown. The total numbers of analyzed animals are given in parentheses. Data were pooled from four independent experiments.

and mock challenged mice at day 9 p.i. ($p < 0.005$–0.001 vs. naive; **Supplemental Figure S7**), which also held true for pulmonal IFN-γ, TNF, MCP-1, and IL-10 concentrations ($p < 0.005$–0.001 vs. naive; **Supplemental Figure S8**).

Hence, Psae challenge during subacute ileitis of hma mice did not result in further increased pro-inflammatory cytokine secretion in extra-intestinal organs, but led to a dampened anti-inflammatory IL-10 response in the liver.

Systemic Pro-Inflammatory Mediator Responses Upon MDR *P. aeruginosa* Challenge of Mice With a Human Gut Microbiota Suffering From Subacute Ileitis

In the following we measured systemic cytokine secretion upon Psae challenge of hma mice during subacute ileitis. At day 9 p.i., increased pro-inflammatory mediators such as IFN-γ, TNF, and NO could be assessed in the spleen derived from mice of either cohort ($p < 0.001$; **Supplemental Figure S9**). In corresponding serum samples, increased IFN-γ, TNF, IL-6, MCP-1, and IL-10 concentrations were determined at day 9 p.i. ($p < 0.05$–0.001; **Figure 9**). Strikingly, even higher TNF and IL-6 levels could be measured in serum samples taken from Psae as compared to mock challenged hma mice with ileitis ($p < 0.01$; **Figures 9 B,C**). Of note, no viable bacteria that might have translocated from the intestinal tract to extra-intestinal and systemic tissue sites during subacute ileitis could be isolated from liver, kidney, lung, heart, spleen, or cardiac blood (not shown). Hence, Psae challenge of hma mice with induced subacute ileitis resulted in an augmented systemic pro-inflammatory cytokine response.

DISCUSSION

In recent years the global emergence of MDR Gram-negative species including Psae in humans as well as in livestock animals has come more and more into the focus of awareness (11, 30). In fact, the increasing prevalence of severe infections caused by MDR Gram-negative bacteria which allow for only very limited options to combat disease contributing to high fatality rates, has not only gained access to the conscience of medical professionals, but also to (health care) politicians as well as to the general public.

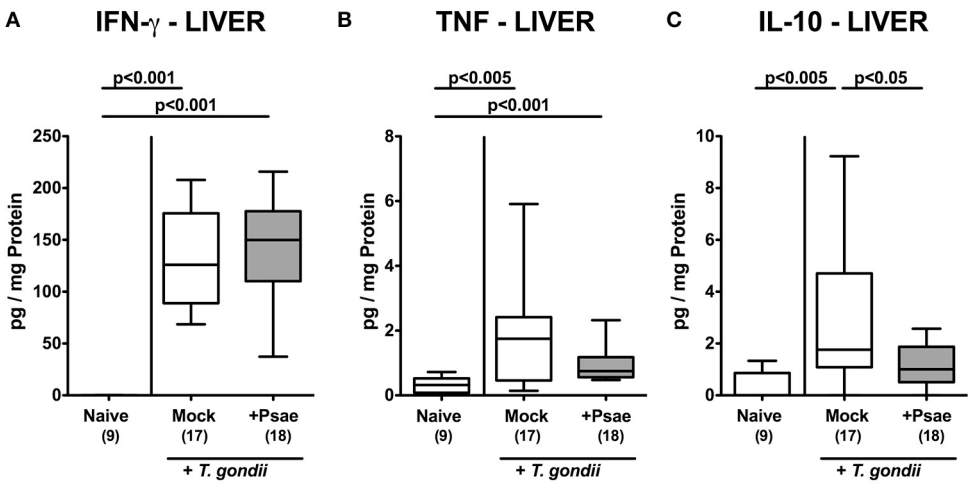

FIGURE 8 | Pro- and anti-inflammatory mediator responses in the liver following peroral MDR *P. aeruginosa* challenge of mice with a human gut microbiota suffering from subacute ileitis. At day 5 following subacute ileitis induction, mice with a human gut microbiota were either perorally challenged with MDR *P. aeruginosa* (+Psae, dissolved in PBS) or with PBS alone (Mock). Uninfected mice with a human gut microbiota but without ileitis served as control animals (Naive). 96 h following Psae challenge, **(A)** IFN-γ, **(B)** TNF, and **(C)** IL-10 concentrations were determined in hepatic *ex vivo* biopsies. Box plots represent the 75th and 25th percentiles of the median (black bar inside the boxes). The total range and significance levels determined by one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are shown. The total number of analyzed animals are given in parentheses. Data were pooled from four independent experiments.

In consequence and of utmost importance, the WHO rated MDR Gram-negative species such as Psae as serious global threat to human health recently (4).

In healthcare settings, the risk of a patient for MDR Psae acquisition increases with the length of the hospital stay, most likely due to spread of the opportunistic pathogen from external sources such as contaminated surfaces and medical devices and/or via smear infection from body fluids or fecal samples derived from colonized/infected patients (5, 6, 31). Particularly information regarding prevalence of human intestinal Psae carriage are scarce, however, and range from 0 to 24% in healthy individuals (31–40), hence speaking against Psae as part of the commensal gut microbiota. One of the key factors facilitating stable intestinal (opportunistic) pathogenic colonization including Psae in mice and men, however, is antibiotic pre-treatment compromising the integrity of the complex gut microbiota, which subsequently provides open niches for the acquired bacteria and hence, potential internal sources for MDR Psae-associated infections lateron (8, 9, 41, 42). In fact, fecal Psae carriage before ICU admission has been shown to be associated with an ∼15-times increased risk for subsequent Psae-induced nosocomial infection (7), which might even more likely mount in a fatal outcome, given that particularly antibiotics-treated ICU patients with concomitant severe comorbidities including immunosuppression are prone to MDR Psae infection arising from the indigenous gut microbiota (3, 11, 43). It is therefore crucial to identify distinct risk factors including immunopathological conditions that might facilitate MDR Psae acquisition by the vertebrate host. Recent studies report higher abundances of Psae in the GIT of patients with underlying intestinal inflammatory diseases such as irritable bowel diseases or ulcerative colitis (40, 44). Interestingly, in our recent study MDR Psae could not only stably colonize the gut, but also induce overt inflammatory immune responses in intestinal, extra-intestinal and even systemic compartments upon peroral challenge of healthy WT mice that were lacking a gut microbiota due to preceding broad-spectrum antibiotic treatment (13). This was also the case, when (with respect to the gut microbiota) "humanized" and clinically uncompromised WT mice were subjected to the identical clinical MDR Psae strain (14).

In the present study, we had challenged hma mice with the opportunistic pathogen after subacute ileitis induction following peroral low-dose *T. gondii* infection—a non-lethal small intestinal inflammation model that has been established by us recently (19). Four days upon peroral application of a rather "supra-physiological" dose of 10^9 CFU, Psae could be isolated from the intestinal tract in approximately one third of hma mice with subacute ileitis and if so, in relatively low counts of $<10^5$ CFU per g luminal sample. In our previous study, intestinal MDR Psae could be cultured in up to 78% of healthy hma mice (i.e., without underlying intestinal inflammatory conditions) 4 weeks following peroral challenge with comparable loads (14). In line with results obtained from our mouse studies, fecal Psae counts were up to 5 log orders of magnitude lower in healthy human volunteers as compared to the bacterial counts within the oral suspension 6 days before (12).

Remarkably, irrespective of the (opportunistic) pathogenic bacterial carrier status, Psae-challenged mice appeared to display more pronounced clinical/macroscopic and microscopic features of subacute ileitis. Whereas, 72.2% of mice from the Psae cohort displayed macroscopic and/or microscopic abundance of blood in their feces 4 day after Psae challenge, this was only the case in 58.8% of mock counterparts. In support, the histopathological

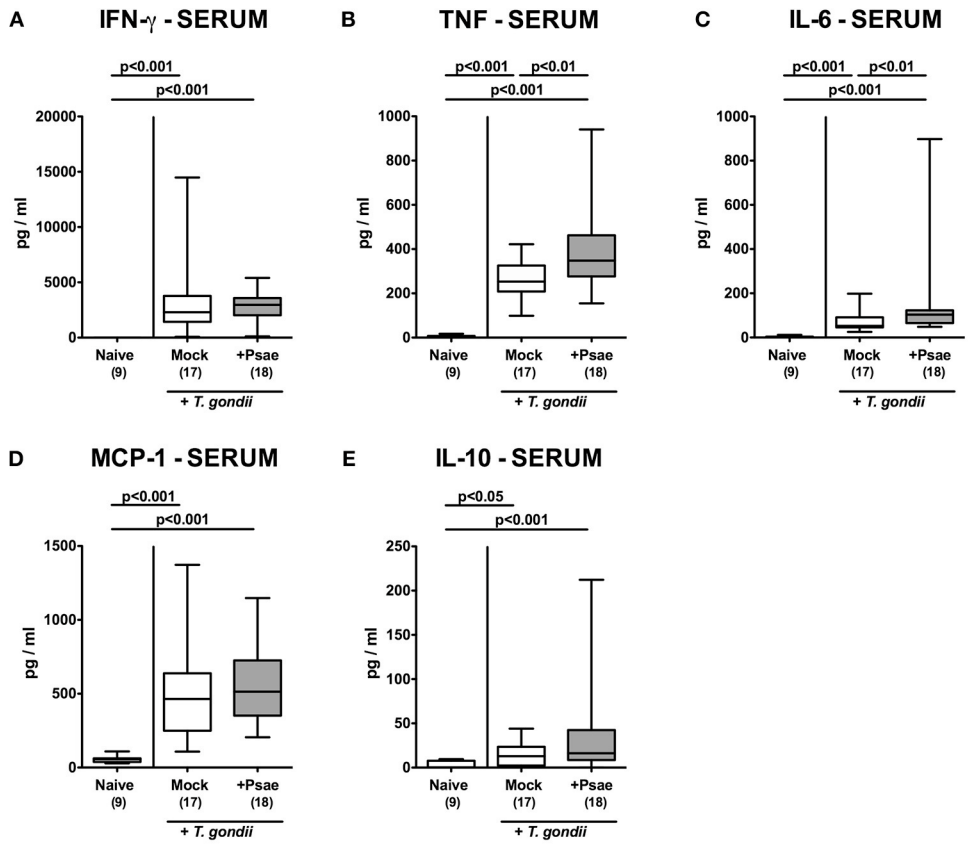

FIGURE 9 | Systemic pro- and anti-inflammatory mediator responses following peroral MDR *P. aeruginosa* challenge of mice with a human gut microbiota suffering from subacute ileitis. At day 5 following subacute ileitis induction, mice with a human gut microbiota were either perorally challenged with MDR *P. aeruginosa* (+Psae, dissolved in PBS) or with PBS alone (Mock). Uninfected mice with a human gut microbiota but without ileitis served as control animals (Naive). 96 h following Psae challenge, **(A)** IFN-γ, **(B)** TNF, **(C)** IL-6, **(D)** MCP-1, and **(E)** IL-10 concentrations were determined in serum samples. Box plots represent the 75th and 25th percentiles of the median (black bar inside the boxes). The total range and significance levels determined by one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are shown. The total number of analyzed animals are given in parentheses. Data were pooled from four independent experiments.

changes in the ileal mucosa tended to be more severe in the Psae cohort as compared to the mock controls, given that individual mice exhibited even virtually fatal ileal mucosal changes such as transmural necrosis 4 days post Psae challenge, which could not be observed in the mock counterparts. The relatively high standard deviation in histopathological scores within the MDR Psae cohort points toward (yet unknown) distinct host factors that might determine the individual outcome of subacute ileitis upon Psae infection.

Furthermore, Psae induced a marked influx of T lymphocytes into the mucosa and lamina propria of both, the ileum and the colon that was accompanied by pronounced apoptotic responses in the ileal epithelia. Remarkably, the marked Psae-induced T cell responses were not restricted to the intestinal tract, but could also be observed in extra-intestinal compartments such as liver, kidney, lungs, and even the heart. In the liver, significant Psae-induced apoptotic changes could be further assessed and were accompanied by less distinct secretion of the anti-inflammatory cytokine IL-10 as compared to mock control mice. Strikingly, Psae challenged hma mice with subacute ileitis displayed even more pronounced systemic pro-inflammatory cytokine responses versus mock counterparts as indicated by higher TNF and IL-6 serum concentrations in the former as compared to the latter. In this context we were, however, not able to detect any viable bacteria originating from the gut microbiota including Psae that had translocated to extra-intestinal including systemic compartments. Nevertheless, it cannot be excluded that soluble bacterial factors including lipopolysaccharide derived from Gram-negative intestinal commensals or from MDR Psae might have found access to the circulation evoking systemic pro-inflammatory immune responses.

In support of our actual results, we demonstrated previously that peroral MDR Psae application to conventionally colonized IL-$10^{-/-}$ mice suffering from chronic colitis aggravated the outcome of the underlying intestinal immunopathological disease (16), again not only on the local (i.e., intestinal) level, but also beyond (i.e., in extra-intestinal and even systemic compartments).

Another recent study applying a lethal acute ileitis model induced by peroral high-dose (i.e., >50 cysts) *T. gondii* infection of hma mice revealed that the underlying acute gut inflammation facilitated Psae colonization (15). The severity

of the gut inflammation model was, however, far too acute to decipher additional Psae induced inflammatory sequelae further accelerating the underlying (already hyper-acute) immunopathology (15).

Nevertheless, given the multitude of so far known Psae virulence factor it is not surprising that intestinal Psae carriage in health and disease mounts in immune responses accelerating the underlying disease. The *Pseudomonas* lipid A moiety, for instance, is part of the Gram-negative bacterial cell wall constituent lipopeptide and able to activate NFκB in a Toll-like receptor (TLR)−4 dependent fashion (45), which subsequently leads to recruitment of innate immune cells to the infection site, further perpetuating host immune responses in order to combat the (opportunistic) pathogenic challenge (1). In support, our previous studies revealed that, in fact, TLR-4 was involved in differentially mediating MDR Psae associated intestinal and extra-intestinal immune responses in IL-10$^{-/-}$ mice (46, 47).

The observed Psae induced immunopathological sequelae in hma mice with subacute ileitis -irrespective of the intestinal carrier status—are supported by our results derived from *Campylobacter jejuni* infection experiments indicating that a pathogen does not necessarily needs to be a permanent member of the intestinal ecosystem to induce pro-inflammatory responses (26, 48–50). More importantly, it is rather the initial hit set by the (opportunistic) pathogen that tips the balance toward immunopathology within the "ménage à trois" of (opportunistic) pathogens, commensal gut microbiota and host immunity (47, 48).

We further assessed whether Psae application resulted in changes of the gut microbiota composition applying culture-independent methods, but could neither find any significant and biologically relevant Psae-induced changes in the luminal microbiota of the inflamed ileum, nor in the co-affected colon. One might, in fact, argue that the observation period of 4 days was far too short for overt changes in the intestinal ecosystem. Nevertheless, our actual results are supported by our previous study where even an intestinal carriage of MDR Psae by conventional IL-10$^{-/-}$ mice with chronic colitis for 6 weeks was not associated with significant changes in the gut microbiota composition as assessed by both, culture and molecular analyses (16).

In conclusion, our actual and previous studies revealed that peroral exposure to MDR Gram-negative opportunistic pathogenic species including Psae might worsen the outcome of underlying inflammatory conditions within the gut. In ongoing studies we are currently exploring antibiotics-independent factors preventing the susceptible host at risk including individuals suffering from inflammatory intestinal comorbidities from Psae-carriage and induced inflammation.

AUTHOR CONTRIBUTIONS

MH: designed and performed experiments, analyzed data, wrote paper; UE and AG: performed experiments, analyzed data, co-edited paper; AK: analyzed data, co-edited paper; SB: provided advice in design and performance of experiments, co-edited paper.

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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.2019.00049/full#supplementary-material

Supplemental Figure S1 | Experimental setup**.** The gut microbiota was depleted in conventional mice by broad-spectrum antibiotic treatment for 8 weeks. Three days before human fecal microbiota transplantation (FMT) the antibiotic cocktail was replaced by sterile tap water to assure antibiotic washout. Microbiota depleted animals were then subjected to human FMT on three consecutive days. In order to guarantee proper establishment of the complex human gut microbiota within the murine host, mice were kept for 10 days before subacute ileitis induction. At day 0, human microbiota associated (hma) mice were perorally subjected to low dose (i.e., one cyst) infection with *T. gondii* infection. 5 days later (d+5), hma mice with induced subacute ileitis were perorally challenged with a multi-drug resistant (MDR) *P. aeruginosa* (Psae) strain and followed up for another 4 days (d+9; necropsy).

Supplemental Figure S2 | Microbiota composition of human donor feces**.** Before human fecal microbiota transplantation of secondary abiotic mice on three consecutive days, main intestinal bacterial groups were quantitatively assessed in human fecal donor suspensions. 16S rRNA of the main intestinal bacterial commensals including enterobacteria (EB), enterococci (EC), lactobacilli (LB), bifidobacteria (Bif), *Bacteroides / Prevotella* species (B/P), *Clostridium coccoides* group (Clocc), *Clostridium leptum* group (Clept), and the total eubacterial load (TL) were analyzed by quantitative RT-PCR and expressed as gene numbers per ng DNA. Data shown are representative for four independent experiments. Total range as well as box plots representing the 75th and 25th percentiles of the median (black bar inside the boxes) are shown.

Supplemental Figure S3 | Representative photomicrographs depicting apoptotic and proliferating epithelial cell as well as immune cell responses in the ileum and colon following peroral MDR *P. aeruginosa* challenge of mice with a human gut microbiota suffering from subacute ileitis. At day 5 following subacute ileitis induction, mice with a human gut microbiota were either perorally challenged with MDR *P. aeruginosa* (ILE, Psae; dissolved in PBS) or with PBS alone (ILE, Mock). Uninfected mice with a human gut microbiota but without ileitis served as control animals (Naive). Representative photomicrographs out of four independent experiments illustrate the average numbers of apoptotic (Casp3+; **A,E**) and proliferating (Ki67+; **B,F**) epithelial cells as well as of T lymphocytes (CD3+; **C,G**) and regulatory T cells (FOXP3+; **D,H**) in at least six high power fields (HPF) that had been quantitatively assessed in ileal **(A–D)** and colonic **(E–H)** paraffin sections 96 h following Psae challenge applying *in situ* immunhistochemistry (100x magnification; scale bar: 100 μm).

Supplemental Figure S4 | Intestinal pro-inflammatory mediator responses following peroral MDR *P. aeruginosa* challenge of mice with a human gut microbiota suffering from subacute ileitis. At day 5 following subacute ileitis

induction, mice with a human gut microbiota were either perorally challenged with MDR *P. aeruginosa* (+Psae, dissolved in PBS) or with PBS alone (Mock). Uninfected mice with a human gut microbiota but without ileitis served as control animals (Naive). 96 h following Psae challenge, IFN-γ concentrations were measured in *ex vivo* biopsies derived from the **(A)** ileum, **(B)** colon, and **(C)** mesenteric lymph nodes (MLN). In addition, **(D)** TNF and **(E)** nitric oxide levels were determined in MLN *ex vivo* biopsies. Box plots represent the 75th and 25th percentiles of the median (black bar inside the boxes). The total range and significance levels determined by one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are shown. The total numbers of analyzed animals are given in parentheses. Data were pooled from four independent experiments.

Supplemental Figure S5 | Representative photomicrographs depicting apoptotic epithelial cell as well as immune cell responses in the liver and kidney following peroral MDR *P. aeruginosa* challenge of mice with a human gut microbiota suffering from subacute ileitis. At day 5 following subacute ileitis induction, mice with a human gut microbiota were either perorally challenged with MDR *P. aeruginosa* (ILE, Psae; dissolved in PBS) or with PBS alone (ILE, Mock). Uninfected mice with a human gut microbiota but without ileitis served as control animals (Naive). Representative photomicrographs out of four independent experiments illustrate the average numbers of apoptotic (Casp3+; **A,D**) epithelial cells as well as of T lymphocytes (CD3+; **B,E**) and regulatory T cells (FOXP3+; **C,F**) in at least six high power fields (HPF) that had been were quantitatively assessed in hepatic **(A–C)** and renal **(D–F)** paraffin sections 96 h following Psae challenge applying *in situ* immunhistochemistry (100x magnification; scale bar: 100 μm).

Supplemental Figure S6 | Representative photomicrographs depicting apoptotic epithelial cell as well as immune cell responses in the lung and heart following peroral MDR *P. aeruginosa* challenge of mice with a human gut microbiota suffering from subacute ileitis. At day 5 following subacute ileitis induction, mice with a human gut microbiota were either perorally challenged with MDR *P. aeruginosa* (ILE, Psae; dissolved in PBS) or with PBS alone (ILE, Mock). Uninfected mice with a human gut microbiota but without ileitis served as control animals (Naive). Representative photomicrographs out of four independent experiments illustrate the average numbers of apoptotic (Casp3+; **A,D**) epithelial cells as well as of T lymphocytes (CD3+; **B,E**) and regulatory T cells (FOXP3+; **C,F**) in at least six high power fields (HPF) that had been were quantitatively assessed in pulmonal **(A–C)** and cardiac **(D–F)** paraffin sections 96 h following Psae challenge applying *in situ* immunhistochemistry (100x magnification; scale bar: 100 μm).

Supplemental Figure S7 | Cytokine responses in the kidneys following peroral MDR *P. aeruginosa* challenge of mice with a human gut microbiota suffering from subacute ileitis. At day 5 following subacute ileitis induction, mice with a human gut microbiota were either perorally challenged with MDR *P. aeruginosa* (+Psae, dissolved in PBS) or with PBS alone (Mock). Uninfected mice with a human gut microbiota but without ileitis served as control animals (Naive). 96 h following Psae challenge, **(A)** IFN-γ, **(B)** TNF, **(C)** MCP-1, and **(D)** IL-6 concentrations were determined in renal *ex vivo* biopsies. Box plots represent the 75th and 25th percentiles of the median (black bar inside the boxes). The total range and significance levels determined by one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are shown. The total numbers of analyzed animals are given in parentheses. Data were pooled from four independent experiments.

Supplemental Figure S8 | Cytokine responses in the lungs following peroral MDR *P. aeruginosa* challenge of mice with a human gut microbiota suffering from subacute ileitis. At day 5 following subacute ileitis induction, mice with a human gut microbiota were either perorally challenged with MDR *P. aeruginosa* (+Psae, dissolved in PBS) or with PBS alone (Mock). Uninfected mice with a human gut microbiota but without ileitis served as control animals (Naive). 96 h following Psae challenge, **(A)** IFN-γ, **(B)** TNF, **(C)** MCP-1, and **(D)** IL-10 concentrations were determined in pulmonal *ex vivo* biopsies. Box plots represent the 75th and 25th percentiles of the median (black bar inside the boxes). The total range and significance levels determined by one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are shown. The total numbers of analyzed animals are given in parentheses. Data were pooled from four independent experiments.

Supplemental Figure S9 | Pro-inflammatory mediator responses in the spleen following peroral MDR *P. aeruginosa* challenge of mice with a human gut microbiota suffering from subacute ileitis. At day 5 following subacute ileitis induction, mice with a human gut microbiota were either perorally challenged with MDR *P. aeruginosa* (+Psae, dissolved in PBS) or with PBS alone (Mock). Uninfected mice with a human gut microbiota but without ileitis served as control animals (Naive). 96 h following Psae challenge, **(A)** IFN-γ, **(B)** TNF, and **(C)** nitric oxide concentrations were determined in splenic *ex vivo* biopsies. Box plots represent the 75th and 25th percentiles of the median (black bar inside the boxes). The total range and significance levels determined by one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are shown. The total numbers of analyzed animals are given in parentheses. Data were pooled from four independent experiments.

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REVIEW
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Gut Microbiome as Target for Innovative Strategies Against Food Allergy

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The dramatic increase in food allergy prevalence and severity globally requires effective strategies. Food allergy derives from a defect in immune tolerance mechanisms. Immune tolerance is modulated by gut microbiota function and structure, and microbiome alterations (dysbiosis) have a pivotal role in the development of food allergy. Environmental factors, including a low-fiber/high-fat diet, cesarean delivery, antiseptic agents, lack of breastfeeding, and drugs can induce gut microbiome dysbiosis, and have been associated with food allergy. New experimental tools and technologies have provided information regarding the role of metabolites generated from dietary nutrients and selected probiotic strains that could act on immune tolerance mechanisms. The mechanisms are multiple and still not completely defined. Increasing evidence has provided useful information on optimal bacterial species/strains, dosage, and timing for intervention. The increased knowledge of the crucial role played by nutrients and gut microbiota-derived metabolites is opening the way to a post-biotic approach in the stimulation of immune tolerance through epigenetic regulation. This review focused on the potential role of gut microbiome as the target for innovative strategies against food allergy.

Keywords: immune tolerance, gut microbiota, mediterranean diet, dysbiosis, probiotics, gut microbiota metabolites, short chain fatty acids, butyrate

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INTRODUCTION

The Changing Scenario of Food Allergy

Food allergy (FA) is one of the most common allergic disorders in the pediatric age, and it has been considered as a global health problem, particularly in industrialized world (1). During the last two decades, studies have suggested that the epidemiology of FA has shown a dramatic increase in the prevalence, severity of clinical manifestations and risk of persistence into later ages, leading to an increase in medical visits, hospital admissions, treatments, burden of care on families, and economic impact, with an increase of costs for the families and healthcare system (2–4). According to the most recent epidemiological data, time trend analysis showed up to a 7-fold increase in hospital admissions for food severe allergic reactions in children in the

UK, USA, Italy and Australia over the last 10 years (5–10). More than 170 foods have been identified as triggers of FA, such as tree nuts, eggs, peanuts, fish, shellfish, milk, wheat, soy, and seeds, with national and geographical variations concerning the most common FA (1, 10–15).

New Insights in the Pathogenesis of FA

FA derives from a breakdown of immune tolerance to dietary antigens (16). Immune tolerance mechanisms involved the activation of dietary antigens specific regulatory T cell (Tregs) (17). Current knowledge suggests that the epidemiology of FA may be influenced by epigenome-genome-environment interactions leading to an alteration of immune system function (18, 19). To stabilize or fall the prevalence of FA, new and innovative strategies to reduce FA incidence are required. Many factors have been postulated to contribute to the onset of FA. The multiple immutable risk factors that could influence FA onset include male sex, ethnicity (increased risk among Asian and African Americans children), and genetics (familial risk, human leukocyte antigen (HLA), and specific genes) (2, 20–25). In addition, there are other modifiable factors that can be potentially targeted to reduce or prevent FA. These factors are related (mode of delivery, breast milk, use of antibiotics or gastric acidity inhibitors, use of antiseptic agents, rural environment, junk food-based and/or low-fiber/high-fat diet, consumption of unpasteurized milk or fermented foods, exposure to pets), or unrelated (comorbid atopic dermatitis, timing and route of exposure to foods, reduced consumption of omega-3-polyunsaturated fatty acids or vitamin D insufficiency, antioxidants,) to an influence on gut microbiome development and function (26–40) (**Figure 1**).

Clinical Consequences of Gut Microbiome Dysbiosis in Children With FA

Many subjects with FA naturally outgrow it over time. Cow's milk allergy (CMA), hen's egg allergy and wheat allergy resolve in ∼50% of children by the age of 5–10 years. Other FAs (including peanuts, tree nuts, fish) have low rates of resolution and are considered persistent (41). In addition, many forms of FA, may be related with later development of other allergic manifestations such as oculorhinitis, atopic dermatitis, asthma, and urticaria (the so called "Atopic March") (42), as well as other diseases such as functional gastrointestinal disorders (FGIDs) (30, 43), inflammatory bowel diseases (IBD) (44), and psychiatric disorders, such as autistic spectrum disorders (ASD) attention deficit hyperactivity disorder (ADHD), and obsessive-compulsive disorder (OCD) (45). The pathogenesis of these events is still largely unknown, but increasing evidence suggest the hypothesis that a perturbation of gut microbiome, leading to alterations in immune system and gut-brain axis, could influence the occurrence of FA and FA-related conditions later in life (**Figure 2**).

Abbreviations: FA, food allergy; CMA, cow's milk allergy; EHCF, extensively hydrolyzed casein formula; LGG, *Lactobacillus rhamnosus* GG; OIT, oral food immunotherapy; SU, sustained unresponsiveness; PBMCs, peripheral blood mononuclear cells; BLG, β lactoglobulin; OVA, ovalbumin; LAB, lactic acid bacteria; NDC, Non-digestible dietary carbohydrates; SCFAs, short chain fatty acids; Tregs, regulatory T cells; DCs, dendritic cells; Kyn, kynurenine; AhR, arylhydrocarbonreceptor; IPA, indole 3-propionic acid; I3A: indole-3-aldehyde; I3C, indole-3-carbinole.

Gut Microbiome Features in FA

Investigating the Metagenomic and Metabolomics Features of Gut Microbiome

The knowledge and awareness of the roles played by gut microbiome and metabolites in the balance between health and disease is rapidly increasing. This is mainly due to advances in technology and the availability of high-sensitivity means to study microbial communities in any type of ecosystem. It is important for the clinicians and researchers dedicated to the FA field to know potential and limits of these technologies to better understand the value and significance of the findings reported in literature. **Box 1** summarizes terminology for gut microbiota-based investigations in FA.

Due to the power of genome DNA sequencing, we have learned much about the composition of gut microbial communities. In addition, the potential of transcriptomics, proteomics, and metabolomics are enlarging our understanding of the gut microbiota role in human health. Until the 1990s, knowledge of the gut microbiome was limited because the structure of gut microbiota was characterized using bacteriological culture. In the last decade, the composition of the gut microbiota was described by next generation sequencing of 16S ribosomal RNA genes. This is increasing the amount of information that can be retrieved by studying metagenomes from human samples, with the capability to infer the abundance of genes and potential metabolic pathways that characterize a microbial community. It is possible to describe the taxonomic composition of the microbiota and also to study the potential functions in a given system. Such methodological background is fundamental to investigate associations between microbiota structure and health as well as other environmental factors (46) and also to observe the changes of the gut microbiota in response to disease or perturbations in diet or lifestyle. An advanced technique to investigate gut microbiota at deep level is shotgun sequencing that represents a massive sequencing of the whole genome. Shotgun sequencing involves DNA random fragmentation, sequencing of these fragments and reconstruction of overlapping sequences to assemble them into a continuous sequence (47). Metabolomics represents one of the meta-omic approaches to study gut microbiota functions. Metabolomics uses high throughput techniques to characterize and quantify small molecules in several biofluids, such as feces, urine, plasma, serum, and saliva (48). The use of metabolomics is considered a powerful top-down systems biology approach, and it is essential to reveal the genetic-environment-health relationship, as well as the clinical biomarkers of diseases (49). Currently, the rapid development of several analytical platform, including liquid chromatography (LC), gas chromatography mass spectrometry (GC- MS), high-pressure LC (HPLC), ultra-pressure LC (UPLC), electrophoresis (CE) coupled to mass spectrometry (MS), Fourier transform infrared spectroscopy (FTIR), ion cyclotrone resonance-FT (ICR-FT), capillary and nuclear, and proton nuclear magnetic resonance spectroscopy (NMR-1H-NMR),

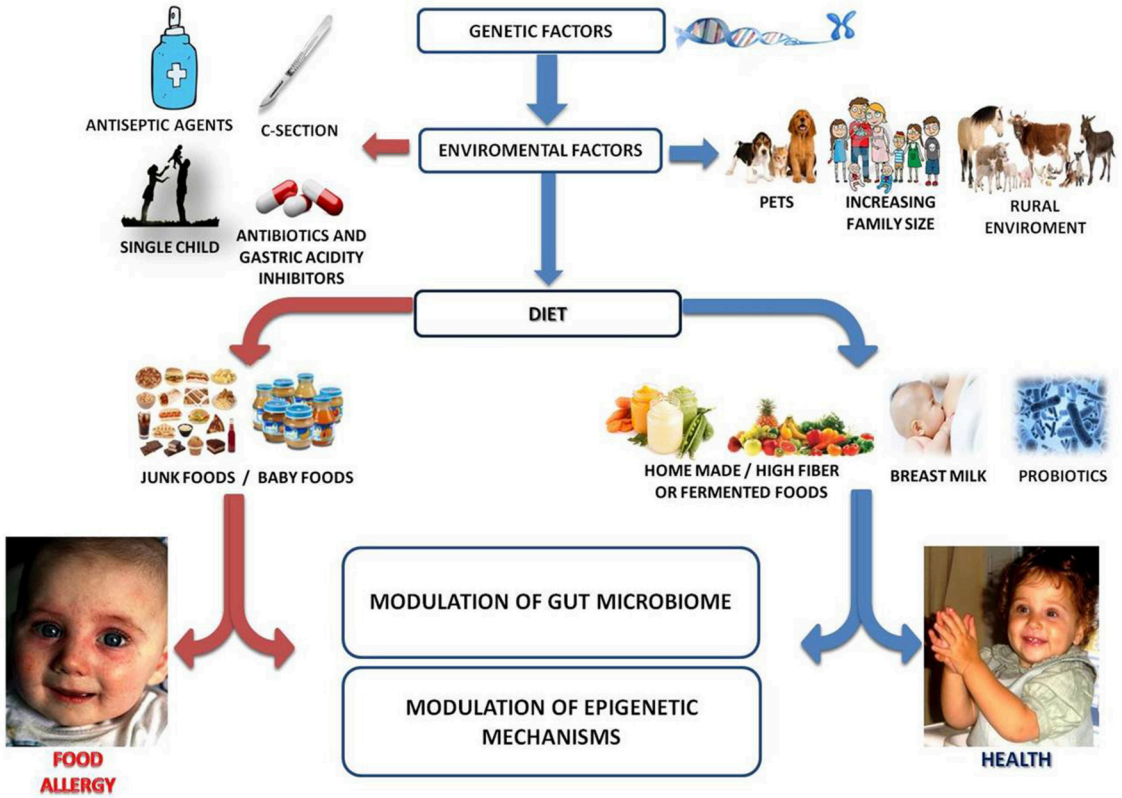

FIGURE 1 | Gut microbiome as a target of intervention against food allergy. Several genetic, environmental, and dietary factors could modulate the gut microbiome-immune system axis influencing the occurrence of FA. For instance, increased family size, exposure to pets and/or rural environment, healthy diet (full of fibers, fermented foods, antioxidants, omega-3), breastfeeding and use of probiotics are associated with protection to FA. Conversely, C-section, prenatal, and early-life exposure to antibiotics/gastric acidity inhibitors/antiseptic agents, unhealthy diet (low fibers/high saturated fats and junk foods) may increase the risk for the development of FA. All these environmental factors act mainly on a modulation of gut microbiota structure and function which in turn could be responsible for the epigenetic regulation of genes involved in immune tolerance.

allowed to better define bacteria related-metabolites and their metabolic pathways (50). **Box 2** summarizes techniques used to investigate the gut microbiota metagenomic and metabolomic features. Gut microbiota metabolomic features are still largely unexplored. Metabolomics will provide important insides in the pathogenesis of FA. In this light, preliminary data available on short chain fatty acids (SCFA) profile are opening new perspective of intervention (see below). What is needed is a transition from descriptive research to understanding the ways the microbiome interacts with the host and plays a role in health and disease. In this frame, controlled clinical interventions are of utmost importance to establish microbiota causative involvement and are the basis to implement approaches of personalized medicine (51, 52). The study of the relationship between microbiome and FA may begin with association and be translated to causation and clinical practice with appropriate advances in knowledge. Wide screening of microbial diversity in gut microbiome of patients with a sure diagnosis of FA, including a well-matched control population, may identify useful signatures in the microbiome that are specific for certain types of FA (53). If the wide screening included cohorts of patients with different dietary style or ethnicity, the common microbial signatures would be even stronger and provide a solid indication of the microbial biomarkers of FA. Further mapping of the genomic features associated with FA may be inferred by metagenomics and metabolomics, which may provide information on the functional microbial signatures associated with FA.

Biomarker strains or defined microbial systems may be tested in gnotobiotic or humanized animal models to observe the development of the disease, and beneficial vs. detrimental microbial metabolites can be recognized and used as final targets of microbiome-targeted personalized interventions. The identification of bacterial metabolites that positively affect the immune tolerance network, may be an interesting strategy against FA using a post-biotic approach.

Evidence on Gut Microbiome Dysbiosis in FA

Mounting evidence indicates that gut microbiome dysbiosis early in life represents a critical factor underlying FA (26, 27, 54, 55). Experimental data from animal models suggest a link between gut microbiome and the occurrence of FA. Tregs was found reduced in mice treated with antibiotic or

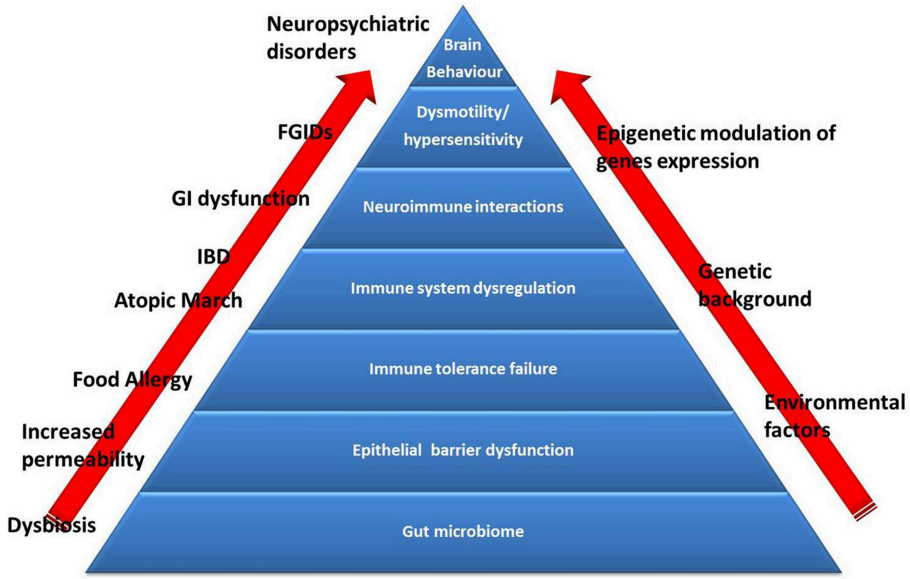

FIGURE 2 | The Food Allergy pyramid. Children with FA present an increased risk to develop other conditions such as allergic disorders (atopic march), inflammatory bowel diseases (IBD), functional gastrointestinal disorders (FGIDs), and neuropsychiatric disorders. Several genetic factors are implicated in the pathogenesis of these conditions, but recent evidence suggest the pivotal role of gut microbiome dysbiosis (induced by environmental factors). Emerging evidence support the hypothesis of dysbiosis as the first hit in the development of alterations in intestinal barrier and immune system function (responsible for the occurrence of FA and atopic march) and dysregulation of the brain-gut endocrine-immune system axis (responsible for the occurrence of FGIDs, IBD, and neuropsychiatric disorders), at least in part through an activation of epigenetic mechanisms.

Box 1 | A brief glossary for a better understanding of the potential of gut microbiota as target against food allergy.

Microbiota	The community of microbes in a particular ecosystem
Microbiome	The sum of micro-organisms, and their total genome capacity, in a particular environment
Operational taxonomic unit	A clusters of micro-organisms, grouped by DNA sequence similarity of a specific taxonomic marker gene. Operational taxonomic units are defined based on the similarity threshold (usually 97% similarity) set by the researcher
Microbiota diversity	A measure of how many different species are distributed in the community
Eubiosis	Healthy balance in a microbial ecosystem
Dysbiosis	A state of imbalance in a microbial ecosystem
Metagenomics	The study of the metagenome; the metagenome is the collective assembly of genomes from an environment (for example, the gut)
Metabolomics	The study of the metabolome; the metabolome is the collective array of metabolites present in a biological sample

in *germ free* mice, with consequent predisposition to allergy development (56–58). Administration of defined Clostridia, or bacteria-derived short-chain fatty acids (SCFA) to *germ free* mice induced an increase of Treg cells number, and reduced allergic response (56, 59–62). The allergy-protective action of Clostridia was also confirmed in the animal model, where a significant protective effect consisting in regulation of innate lymphoid cell function, Foxp3$^+$ Tregs, immunoglobulin (Ig)A and intestinal epithelial permeability was demonstrated (63). A "humanized mice model," created with inoculation of microbiota-derived from human feces, resulted in an increase in Treg cells and a reduction of allergic symptoms (64). The functional role of dysbiosis associated with FA was also revealed by the different capacity of the gut microbiota of allergen-sensitized mice to increase Th2 cells number and IgE responses and to promote allergic sensitization (17).

Unfortunately, data characterizing the gut microbiome of patients affected by FA are still preliminary.

Table 1 summarizes main evidence on FA-associated gut microbiome features. Heterogeneity in study design, used to define the gut microbiome, make it difficult to establish a causal relationship between development of FA and specific bacteria. Despite these limitations, at least four relevant observations on FA-associated gut microbiome can be raised:

- Dysbiosis precedes the FA onset;
- Microbial community structure early in life, particularly in the first 6 months of life, is more relevant in FA development;
- No specific bacterial taxa could be consistently associated with FA onset, with a broad range of microbes that could have positive or negative influence on tolerogenic mechanisms;
- Dysbiosis could influence not only the occurrence, but also the disease course of FA. As suggested by different gut microbiota features comparing children who outgrow FA with patients with persistent form of FA (71).

Recent studies underline the importance of the modulation of gut microbiota through different dietary interventions in pediatric patients with FA. CMA children treated with soy and rice based

BOX 2 | Techniques used to investigate the gut microbiota metagenomic and metabolomic features.

Technique	Description	Advantages	Disadvantages
Metagenomics			
Culture	Isolation of bacteria on selective media	Cheap, semi-quantitative	Labor intensive
qPCR	Amplification and quantification of 16S rRNA. Reaction mixture contains a compound that fluoresces when it binds to double-stranded DNA	Fast,quantitative, Phylogenetic identification	PCR bias, unable to identify unknown species
DGGE/TGGE	Gel separation of 16S rRNA amplicons using denaturant/ temperature	Fast, semi-quantitative, bands can be excised for further analysis	No phylogenetic identification, PCR bias
T-RFLP	Fluorescently labeled primers are amplified and then restriction enzymes are used to digest the 16S rRNA amplicon. Digested fragments separated by gel electrophoresis	Fast, cheap, semi-quantitative	No phylogenetic identification, PCR bias, low resolution
Fish	Fluorescently labeled oligonucleotide probes hybridize complementary target 16S rRNA sequences. When hybridization occurs, fluorescence can be enumerated using flow cytometry	Phylogenetic identification, semi-quantitative, no PCR bias	Dependent on probe sequences—unable to identify unknown species
DNA microarrays	Fluorescently labeled oligonucleotide probes hybridize with complementary nucleotide sequences. Fluorescence detected with a laser	Fast, Phylogenetic identification, semi-quantitative	Cross hybridization, PCR bias, species present in low levels can be difficult to detect
Cloned 16S rRNA gene sequencing	Cloning of full-length 16S rRNA amplicon, Sanger sequencing and capillary electrophoresis	Phylogenetic identification, quantitative	PCR bias, laborious, expensive, cloning bias
Direct sequencing of 16S rRNA amplicons	Massive parallel sequencing of partial 16S rRNA amplicons for example, 454 Pyrosequencing® (Roche Diagnostics GMBH Ltd, Mannheim, Germany) (amplicon immobilized on beads, amplified by emulsion PCR, addition of luciferase results in a chemoluminescent signal)	Fast, Phylogenetic identification, quantitative, identification of unknown bacteria	PCR bias, expensive, laborious
Microbiome shotgun sequencing	Massive parallel sequencing of the whole genome (e.g., 454 pyrosequencing® or Illumina®, San Diego, CA, USA)	Phylogenetic identification, quantitative	Expensive, analysis of data is computationally intense
Metabolomics			
Gas Chromatography Mass Spectrometry (GC-MS)	Thermally stable and volatile compounds are separated by GC and the eluting metabolites are detected by electron-impact (EI) mass spectrometers.	High efficiency, reproducibility and sensitivity	It can only be performed for volatile compounds
Liquid Chromatography Mass Spectrometry (LC)	Allows to separate compounds with little effort in a few pre-analytics steps (compared to GC-MS). The metabolite separation obtained with LC is followed by electro spray ionization (ESI) or atmospheric chemical ionization under pressure (APCI)	Lower temperatures of analysis, and it does not require sample volatility. Sensitivity, specificity, resolving power, and capability to extract additional information about metabolites from their retention time (RT) domain.	
Capillary Electrophoresis Mass Spectrometry (CE)	Offers high-analyte resolution and detect a wider spectrum of (polar) compounds compared to HPLC.	High resolution	It is properly applicable only to charged analytes
Fourier Transform Infrared Spectroscopy (FTIR)	Allows rapid, non-destructive and high-throughput determination of different sample types. This technique allows detecting different molecules, such as lipids and fatty acids (FAs), proteins, peptides, carbohydrates, polysaccharides, nucleic acids.	Ultra-high mass resolution able to distinguish slight variations in a wide number of mass signals, and allowing to obtain the structural identification of new biomarkers	Not high sensitivity and selectivity
Nuclear Magnetic Resonance Spectroscopy (NMR)	It uses the intramolecular magnetic field around atoms in molecules to change the resonance frequency, thus allowing access to details of molecules' electronic structure and obtaining information about their dynamics, reaction state, and chemical environment.	Useful to determine metabolic fingerprints leading to the identification and quantification of compounds in a non-targeted large-scale, in a non-destructive way, and with a high reproducibility	It is a relatively insensitive technique, and can only detect metabolites in high concentrations

formula showed low fecal abundance of *Coriobacteriaceae* and *Bifidobacteriaceae*. Contrarily, *Coriobacteriaceae*, and certainly the genus Collinsella, the major bacteria that metabolized lactose in the gut, resulted increased in CMA children that consumed extensively hydrolyzed formula. In the same study, the authors found that fecal butyrate levels are positive correlated with abundance of *Coriobacteriaceae* (77). We showed that the treatment with extensively hydrolysed casein formula (EHCF)

TABLE 1 | Main gut microbiome features in food allergy.

	OTUs	Diversity	Technology	Main features	References
Björkstén et al. (65) (*n* = 62; FA)	N.R.	N.R.	*Bacterial culture*	↑*Coliforms, S. Aureus* ↓*Lactobacilli, Bifidobacteria*	(65)
Thompson-Chagoyan et al. (66) (*n* = 46:FA)	↑	N.R.	*Bacterial culture*	↑ *Lactobacilli* ↓*Bifidobacteria*	(66)
Thompson-Chagoyan et al. (67) (*n* = 46:FA)	N.R.	N.R.	*Bacterial culture*	↑*C.coccoides*, *Atophium cluster*	(67)
Nakayama et al. (68) (*n* = 11: FA)	=	=	16s rRNA sequencing	↑*Bacteroides,Propionibacterium*, *Klebsiella* ↓*Acinobacterium, Clostridium*	(68)
Ling et al. (69) (*n* = 34: FA)	↓	=	16s rRNA sequencing	↑ *Bacteroidetes*, *Proteobacteria*, *Actinobacteria* ↓*Firmicutes*	(69)
Azad et al. (55) (*n* = 12: FS)	↓	=	16s rRNA sequencing	↓*Enterobacteriaceae*, *Bacteroidaceae*	(55)
Chen et al. (70) (*n* = 23: FS)	N.R.	↓	16s rRNA sequencing	↑*Firmicutes*, *Proteobacteria*, *Actinobacteria* ↓*Veillonella*	(70)
Berni Canani et al. (53) (*n* = 39; FA)	↑	N.R.	16s rRNA sequencing	↑*Ruminococcaceae*, *Lachnospiraceae* ↓*Bifidobacteriaceae*, *Streptococcaceae*, *Enterobacteriaceae*	(53)
Bunyavanich et al. (71) (*n* = 226; FA)	↑	N.R.	16s rRNA sequencing	↑*Bacteroidetes*, *Enterobacter*	(71)
Inoue et al. (72) (*n* = 4: FA)	N.R.	N.R.	16s rRNA sequencing	↑*Lachnospira*, *Veillionella*, *Suterella* ↓*Dorea*, *Akkermansia*	(72)
Kourosh et al. (73) (*n* = 68; FA)	↑	N.R.	16s rRNA sequencing	↑*Oscillobacter valericigenes*, *Lachnocrostidium bolteae*, *Faecalibacterium* sp.	(73)
Fazlollahi et al. (74) (*n* = 141; FA)	N.R.	N.R.	16s rRNA sequencing	↑*Lachnospiraceae*, *Streptococcaceae*, *Leuconostocaceae*	(74)
Dong et al. (75) (*n* = 60; FA)	N.R.	↓	16s rRNA sequencing	↑*Lactobacillaceae*, ↓*Bifidobacteriaceae*, *Ruminococcaceae*	(75)
Berni Canani et al. (76) (*n* = 46; FA)	=	=	16s rRNA sequencing	↑*Bacteroides, Alistipes*	(76)
Diaz et al. (77) (*n* = 27; FA)	N.R.	N.R.	16s rRNA sequencing	↑*Coriobacteriaceae*	(77)

FA, food allergy; FS, sensitization to food antigens; OTUs, operational taxonomic units; N.R., not reported; ↑, increase; ↓, decrease.

containing the probiotic *L. rhamnosus* GG (LGG) in CMA children significantly increased SCFA-producers bacteria and butyrate fecal levels. These effects were associated with immune tolerance acquisition (76).

Targeting Gut Microbiome in FA

The Importance of the Diet-Gut Microbiome Axis

Advances in metagenomics and metabolomics implicate diet and gut microbiome (the diet-gut microbiome axis) as key modulators of the maturation of the immune system. Findings from a recent systematic review further support the relationship between maternal diet during pregnancy and lactation and FA during childhood (78). Diet from conception (maternal diet) up to the first 24 months of age (baby diet), may influence the risk of developing FA (78–81). A recent study suggests that a healthy diet with high levels of fruits, vegetables and home-made foods is associated with less FA at the age of 24 months (82). Several studies have reported that nutrients impact the gut microbiota and the production of bacterial metabolites (83, 84). The Mediterranean diet (MD) is defined as a healthy balanced diet. It is characterized by high consumption of assorted cereals, legumes, fruits, vegetables, olive oil, and nuts; moderate consumption of red wine, poultry and fish, and a lower intake of red meat and sweets. MD during pregnancy and early life has been demonstrated to have a protective role against allergic disease in children (85). These effects could derive from the high intake of non-digestible dietary carbohydrates (NDC), the beneficial fatty acid profile that is rich in omega-3, the high levels of polyphenols, and other antioxidants (86). Non-digestible dietary carbohydrates represent the primary nutrient source for gut bacteria, and their fermentation leads to the production of SCFAs) (53, 87). It has been demonstrated that

reduced availability of NDC lowered the concentration of fiber-degrading bacteria and increased mucin-degrading bacteria (88). High adherence to the MD has been associated with-increased levels of *Prevotella* bacteria and other *Firmicutes* and of SCFAs production (89). The immunomodulatory mechanisms elicited by SCFAs represent one of the strongest connections between diet, gut microbiome and allergic diseases (90). Major SCFAs included acetate, propionate, butyrate, and valerate (87). SCFA-producing bacteria represent a functional group, including *Faecalibacterium prausnitzii* and *Eubacterium rectal*, *Roseburia* are efficient butyrate producers (91). SCFAs are major energy source for colonocytes and influence epigenetically several non-immune (tight junction proteins, mucus production) and immune functions (macrophages, neutrophils, dendritic cells (DCs), T and B cells) involved in the immune tolerance network (92–98). SCFAs interaction with enterocytes are mediated by G-protein coupled receptors, namely GPCRs; GPR41, GPR43, GPR109A, and Olfr78) (99–101). GPR43 and GPR41 are highly expressed by enterocytes (102), whereas immune cells express GPR43 and GPR109A (100, 103–106). Among SCFAs, butyrate exerts a pivotal role in immune tolerance induction. It has been found that SCFAs are able to increase colonic Treg frequency and *in vitro* treatment of colonic Tregs, from germ free mice, with propionate significantly increased *FoxP3* and IL-10 expression, a key cytokine that regulate Treg functions (60). Similarly, it has been demonstrated that butyrate facilitates generation of activated FoxP3$^+$ Treg in mouse model (107).

Butyrate is able to regulate 103$^+$DCs, reducing pro-inflammatory cytokines production and enhancing retinoic acid (RA) expression and subsequent generation of RA-regulated tolerogenic DCs (108). Butyrate promotes B cell differentiation and increases IgA and IgG production (107, 109).

The mechanisms are multiple and involve a strong epigenetic regulation of gene expression through the inhibition of histone deacetylase (HDAC) (60, 110, 111).

Butyrate deficiency has been observed in allergic children (112). Bacteria-produced SCFAs have been studies, has been specifically attributed to butyrate production by spore-forming Clostridiales. An enrichment of butyrate-producing taxa (Clostridia class and *Firmicutes phylum*) has been observed in children with faster CMA resolution (71). Altogether, these data suggest the potential of a "post-biotic" approach, based on the use of SCFAs against FA. In this light, data from our laboratory showed that oral butyrate induces a dramatic inhibition of acute allergic skin response, anaphylactic symptom score, body temperature decrease, intestinal permeability increase, anti-β lactoglobulin (BLG) IgE, IL-4, and IL-10 production in a murine model of CMA, suggesting a protective role of butyrate against FA (113).

We evaluated the direct effects of butyrate on peripheral blood mononuclear cells (PBMCs) from children affected by challenge-proven IgE-mediated CMA. PBMCs were stimulated with BLG in the presence or absence of butyrate. Preliminary results showed that butyrate stimulates IL-10 and IFN-γ production and decreases DNA methylation rate of these two cytokine genes. The same effective butyrate dose induces *FoxP3* demethylation and down-regulation of HDAC6/HDAC9 expression (113, 114).

TABLE 2 | Main preclinical evidences on the probiotics role against food allergy.

Biological effects	Bacterial strains	References
Intestinal barrier maturation	*B. lactis/bifidum*; *L. rhamnosus GG*	(128, 130, 135)
Th1/Th2 response balance: Th1 stimulation	*B. lactis/bifidum/ infantis*; *L. acidophilus/reuteri; L. rhamnosus GG*	(132, 133, 136, 137)
Th1/Th2 response balance: Th2 suppression	*B. bifidum/infantis/longum; L. actobacillus acidophilus/reuteri; L. rhamnosus GG*	(132, 134, 138–140)
Immune system regulation: Tregs development	*B. bifidum/infantis/lactis; L. acidophilus/reuteri/casei; L. rhamnosus GG*	(132, 134, 137)
Increase in B and T cell proliferation with enhanced production of Th1 and regulatory cytokines	*L. acidophilus; L. casei; L. salivarius; L. lactis; B. infantis; B. lactis; B. longum*	(135)
Immune system regulation: tolerogenic DCs development	*B. bifidum; L. reuteri/casei*; *L. rhamnosus GG*	(134, 137, 141, 142)
Suppression of IgE production	*B. bifidum/longum*; *B. lactis Bb-12*; *L. acidophilus*; *L. rhamnosus GG*	(128, 133, 138, 143, 144)
Epigenetic modulation of Th1/Th2 genes expression	*B. breve; L. rhamnosus GG*	(145–147)
Increase in the production of the regulatory cytokine IL-10 by monocytes and dendritic cells; enhance of IFN-γ production by T cells	*L. plantarum; B. adolescentis*	(141, 148, 149)
Increase in the population of CD4$^+$FoxP3$^+$ T cells, up-regulation of FoxP3 and down-regulation of GATA-3	*L. plantarum; B. coagulans*	(145)
Reduction of allergic reaction; reduction of IL-4, IL-5, IL-13 and specific IgE production	*L. rhamnosus GG*	(139)
Improvement of anaphylaxis symptoms and increase of sIgA and CD4$^+$ CD25$^+$ FoxP3Treg cell	*C. butyricum*	(150)

Additional potential mechanisms by which diet could exert pro-tolerogenic effects in the gut are related to the production of immunoregulatory metabolites, which interact with the host immune cells to promote non-responsiveness to innocuous luminal antigens (115). Tryptophan is an essential amino acid, which cannot be synthesized independently by humans; thus, it must be ingested through the diet. A portion of tryptophan is utilized to synthesize protein, and the other portion is catabolized to produce a variety of bioactive compounds, such as kynurenine (Kyn), serotonin and melatonin (84). Tryptophan absorbed by enterocytes directly activates the mTOR pathway by intracellular tryptophan receptors (116, 117). mTOR is known to play an important role in connecting metabolism and the immune system. During an inflammatory process,

tryptophan is metabolized through the Kyn pathway. Kyn is an active metabolite and its biological activity is mediated by aryl hydrocarbon receptor (AhR) (118). The bond of Kyn to AhR receptor lead to the inhibition of DCs maturation and the proliferation of Th17 cells and Treg, increasing IL-22 and IL-10 production (119–122). Indole, indole 3-propionic acid (IPA) and indole-3-aldehyde (I3A) are produced by catabolism of tryptophan through intestinal commensal bacteria. A study demonstrated that strains of *Clostridium cadaveris* and *Peptostreptococcus anaerobius* CC14N metabolize tryptophan to produce IPA. Tryptophan can be also catabolized by lactobacilli to I3A. This metabolite protects gut mucosa against inflammation through AhR recognition (123). Indole-3-carbinole (I3C), an AhR ligand, has been demonstrated to boost immune tolerance in an ovalbumin (OVA)-sensitized mouse model (124). Mice fed I3C showed lower titres of anti-OVA IgG1 antibodies and higher number of $CD103^{+}MHC\text{-}II^{+}$ tolerogenic DCs compared to normal chow-fed control mice (124).

Engineering Gut Microbiome With Probiotics in FA

Immune tolerance is a major therapeutic target in FA. Evidence supports the concept that probiotics, defined as live microorganisms which when ingested in adequate amounts confer a beneficial effect on the host (125), could act at different levels in the immune tolerance network: modulating gut microbiota structure and function (increased production of butyrate) (53); interacting with enterocytes with subsequent modulation of non-immune (gut permeability and mucus thickness) (126–129) and immune tolerogenic mechanisms (stimulation of sIgA and β-defensins production) (130); modulation of cytokine response by immune cells (110–113, 131–134). Main pre-clinical evidence on probiotic activity against

Box 3 | Targeting gut microbiota against FA: a research agenda.

Targets	Possible strategies
Identifying specific gut microbiota features associated with FA	To comparatively analyze metagenomics and metabolomics features of well-characterized populations of patients affected by different types of FA(naive of any dietary treatment) and healthy well-matched controls.
Characterizing the effect of dietary intervention and probiotic therapy	Prospective studies analyzing gut metagenomic and metabolomics changes in well-characterized populations.
Identifying the best probiotic strain to treat FA	Studies on mechanisms action in *in vitro* and in *in vivo* models. Clinical trials with well-characterized probiotic strains and doses involving patients with challenge-proven diagnosis of FA.
Optimizing the post-biotic approach to treat FA	Full characterization of the bio-functional features of gut microbiota metabolites that could be used against FA. Studies on mechanisms action in *in vitro* and in *in vivo* models. Clinical trials with well-characterized products involving patients with challenge-proven diagnosis of FA.

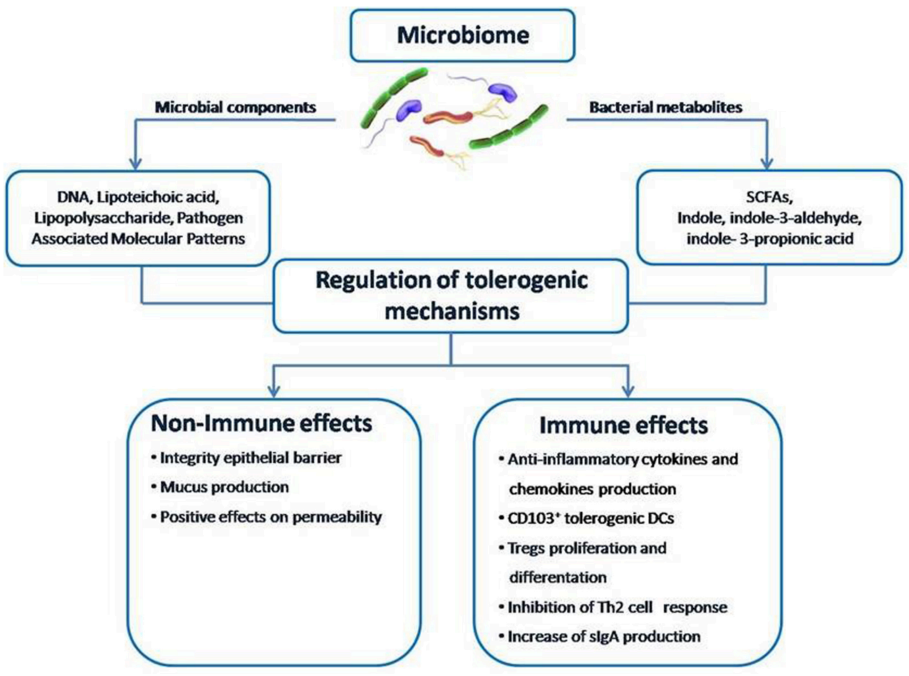

FIGURE 3 | The structure of the gut microbiome-immune system axis. Within the gut microbiome-immune system axis the cross talk between microbes and the immune system may occur directly through microbial components or indirectly through the action of metabolites, such as SCFAs. A positive modulation of this axis can counteract the pathogenesis of FA by promoting epithelial integrity, gut permeability, mucus production, $CD103^{+}$ tolerogenic DCs, Treg differentiation, cytokines production, and sIgA release from plasma cells.

FA are summarized in **Table 2**. In the last decades, a number of experimental investigations have been developed to characterize organisms that could be used to modulate the immune system of patients with FA. Stimulation of human PBMCs with selected probiotic strains is a commonly used experimental tool for the investigation of the effect of these microorganisms on immune cells. The incubation of PBMCs with *L. plantarum* and *B. adolescentis* resulted in an increased production of the regulatory cytokine IL-10 by monocytes and DCs, and to enhanced IFN-γ production by T cells) (138, 148, 149). The addition of a probiotic mixture (*L. casei* W56, *L. lactis* W58, *L. acidophilus* W55, *L. salivarius* W57, *B. infantis* W52, *B. lactis* W18, and *B. longum* W51) to PBMCs from children with FA stimulated an increase of Th1 cells and related cytokines (141). An increase in T and B cells proliferation and a reduction in IgE production, were also observed in PBMCs from children with FA treated for 3 months with the same probiotic mixture (141). Using a 3D co-culture model of intestinal epithelial cells and PBMCs as an *in vitro* model of the intestinal mucosal immune system, Ghadimi et al. demonstrated that the probiotics *B. breve* and LGG inhibit activation of proinflammatory cytokines, IL-23, and IL-17, thereby reducing histone acetylation and simultaneously enhancing DNA methylation (135). The limitation of studying the effect of probiotics *in vitro* lies in the extrapolation of the results of *in vivo* benefits. For that reason, another commonly used experimental tool in this area is based on the use of animal model of FA. Using an OVA mouse model, it was demonstrated that oral administration of *B. infantis* reduced serum OVA-specific IgE, and IgG1 levels and Th2 cytokine release from splenocytes. Moreover, gut microbiota analysis showed that the probiotic-mediated protection was conferred by high abundance of *Coprococcus* and *Rikenella* (151). Different effects of oral administration of *B. coagulans* 09.712, *L. plantarum* 08.923, and *B. infantis* 11.322 in the reduction of Th2-driven intestinal inflammation and other symptoms associated with food-induced anaphylaxis, were demonstrated in a murine model of shrimp allergy (145). In particular, oral supplementation with *B. coagulans* 09.712 and *L. plantarum* 08.923 significantly ameliorates anaphylaxis symptoms and increases the population of $CD4^+$ $CD25^+FoxP3^+$ T cells through mTORC inhibition, FoxP3 upregulation, and GATA-3 downregulation (145). Oral treatment with *C. butyricum* significantly ameliorated anaphylaxis symptoms and increased sIgA and $FoxP3^+$Treg cells in the spleen from BLG-sensitized mice (150). Neonatal monocolonization of germ-free mice by *L. casei* BL2 modulated the allergic sensitization to cow's milk proteins, developed higher IgG responses against caseins, elicited by *L. casei* hydrolysed insoluble caseins into soluble immunogenic peptides (152). Similar results were obtained by others who observed a decrease of concentrations of IgE, IL-4, and IL-13 following administration of *B. infantis* CGMCC313-2 in BLG-sensitized mice (153). Oral administration of VSL#3 (a mixture of *Streptococcus thermophilus* BT01, *B. breve* BB02, *B. longum* BL03, *B. infantis* BI04, *L. acidophilus* BA05, *L. plantarum* BP06, *L. paracasei* BP07, *L. delbrueckii* subsp. *bulgaricus* BD08) to sensitized mice significantly reduces Th2 immune responses and protects against anaphylactic reactions in a mouse model of FA (154). Also, the incubation of mouse spleen cells from sensitized mice with probiotic mixture reduced allergen- stimulated IL-13 and IL-5 production and increased of IFN-γ and IL-10 production (154). An immunoregulatory action by LGG has been demonstrated in a murine model of CMA. LGG administration suppressed Th2 responses, such as reduced hypersensitivity score and lowered serum CMP-specific IgG1, while promoting IFN-γ and CMP-specific IgG2a levels (155). Similar results have been reported by our group in a BLG-sensitized mice model, in which we found that the administration of LGG added to EHCF elicited a significant

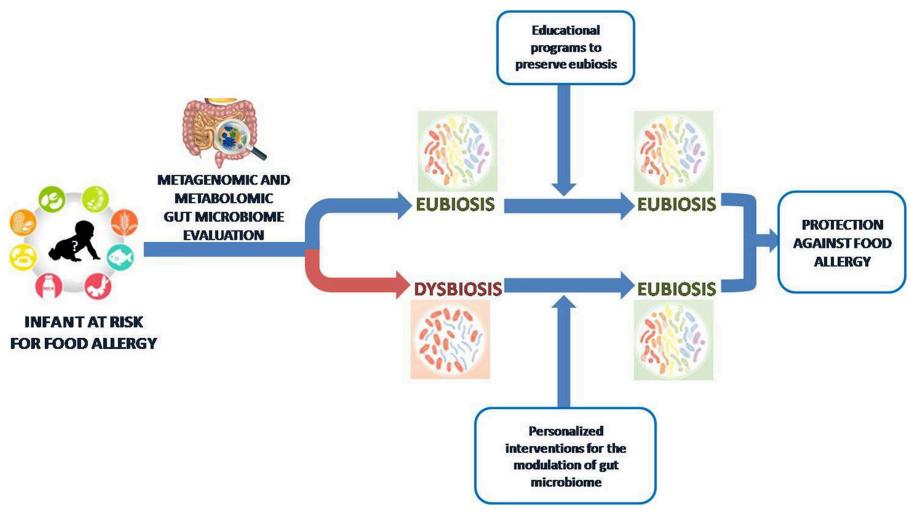

FIGURE 4 | Toward a gut microbiome-based precision medicine against food allergy. We are approaching an era where the metagenomic and metabolomic evaluation of gut microbiota in children at risk for FA will drive personalized intervention to preserve or restore an "eubiosis" state based on nutritional counseling and educational programs.

reduction of allergic reaction, and of IL-4, IL-5, IL-13 and specific IgE production (139).

Clinical studies have investigated the efficacy of selected probiotic strains against FA. The effect appears strain-specific and more evident in the pediatric age group. In a randomized double-blind placebo-controlled trial, it was demonstrated that the administration of *L. casei* CRL431 and *B. lactis* BB12 added to hypoallergenic formula for 12 months did not modulate the rate of immune tolerance acquisition to cow's milk proteins in infants with CMA (140). Using a similar study design, we have demonstrated that EHCF containing the probiotic LGG is able to accelerate immune tolerance acquisition in CMA children. Children (aged 1–12 months), consecutively referred for suspected CMA (IgE- or non-IgE-mediated), but still receiving cow's milk proteins, were evaluated in the study. Subjects were randomly allocated to one of the two groups of dietary interventions: EHCF (control group); and EHCF containing LGG (at least 1.4×10^7 CFU/100 mL; active group). After 12 months, the double-blind placebo- controlled food challenge was negative in 15 of 28 control infants (53.6%) and in 22 of 27 infants receiving EHCF with LGG [(81.5%, $p = 0.027$)] (156). The results were confirmed in a subsequent trial, when the effect of 5 different dietary strategies was investigated: EHCF, EHCF + LGG, partially hydrolyzed rice formula, soy formula or amino acid-based formula, in children affected by IgE- or non-IgE-mediated CMA. After the treatment period of 12 months, the proportion of children acquiring immune tolerance to cow's milk proteins was significantly higher in the group receiving EHCF+LGG (78.9%) than in other groups (157). At the 3-year follow- up of another pediatric cohort, a further confirmation of a greater rate of resolution of IgE-mediated CMA as well as a lower incidence of other atopic manifestations was described after treatment with EHCF+LGG (158). These effects could derive at least in part by a modulation elicited by selected LGG components on immune functions through different pathways including enterocytes, monocytes, mast cells, DCs and Tregs (159–162), and by an expansion of butyrate- producing gut microbiota (53, 76). Accordingly, studies in children with eczema and/or CMA who received EHCF plus LGG showed benefits in decreasing inflammation and gastrointestinal symptoms (163). Probiotics have been also proposed to reinforce the effectiveness of immunotherapy (164). Oral food immunotherapy (OIT) is currently the most investigated approach for persistent FA and it is based on the concept that repeated oral/intestinal exposures to antigens normally leads to tolerance. Randomized double-blind placebo- controlled trial was performed in 62 children with peanut allergy treated with fixed dose of probiotic together with peanut OIT (PPOIT) or placebo once daily for 18 months (165). Sustained unresponsiveness (SU), determined by double blind placebo controlled food challenge (DBPCFC), was achieved in 82.1% of children receiving PPOIT compared with 3.6% of those receiving placebo. PPOIT also induced high rates of resolution (90%) and was associated with reduced skin prick test reactivity, decreased peanut-specific IgE and increased peanut-specific IgG4 levels. No participants withdrawing because of adverse reactions.

No OIT control group was evaluated to determine if the probiotic itself had any effect on SU (165). Further studies are required to evaluate this approach comparing peanut OIT and probiotics with peanut OIT with placebo or probiotic alone.

CONCLUSIONS

Gut microbiome could be a promising target for innovative therapeutic and preventive strategies against FA. The results of the studies are encouraging, but more data are needed to better define the potential of modulating the diet-gut microbiome–immune system axis to fight FA (**Figure 3**). We are approaching a new era in which we can regulate immune system development and function through dietary intervention and measure the clinical impact through gut microbes and their metabolites. Given the current gaps in the investigational approaches and data analysis and interpretation, we need more scientific evidence that can be translated in clinical practice (**Box 3**).

Understanding how nutrients and metabolites, or probiotics could influence gut bacteria communities and the immune system will contribute to building up a precision medicine approach for FA care (**Figure 4**).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Pituitary Adenylate Cyclase-Activating Polypeptide—A Neuropeptide as Novel Treatment Option for Subacute Ileitis in Mice Harboring a Human Gut Microbiota

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The neuropeptide Pituitary adenylate cyclase-activating polypeptide (PACAP) is well-known for its important functions in immunity and inflammation. Data regarding anti-inflammatory properties of PACAP in the intestinal tract are limited, however. In our present preclinical intervention study we addressed whether PACAP treatment could alleviate experimental subacute ileitis mimicking human gut microbiota conditions. Therefore, secondary abioitic mice were subjected to human fecal microbiota transplantation (FMT) and perorally infected with low-dose *Toxoplasma gondii* to induce subacute ileitis on day 0. From day 3 until day 8 post-infection, mice were either treated with synthetic PACAP38 or placebo. At day 9 post-infection, placebo, but not PACAP treated mice exhibited overt macroscopic sequelae of intestinal immunopathology. PACAP treatment further resulted in less distinct apoptotic responses in ileal and colonic epithelia that were accompanied by lower T cell numbers in the mucosa and lamina propria and less secretion of pro-inflammatory cytokines in intestinal *ex vivo* biopsies. Notably, ileitis-associated gut microbiota shifts were less distinct in PACAP as compared to placebo treated mice. Inflammation-ameliorating effects of PACAP were not restricted to the intestines, but could also be observed in extra-intestinal including systemic compartments as indicated by lower apoptotic cell counts and less pro-inflammatory cytokine secretion in liver and lungs taken from PACAP treated as compared to placebo control mice, which also held true for markedly lower serum TNF and IL-6 concentrations in the former as compared to the latter. Our preclinical intervention study provides strong evidence that synthetic PACAP alleviates subacute ileitis and extra-intestinal including systemic sequelae of T cell-driven immunopathology. These findings further support PACAP as a novel treatment option for intestinal inflammation including inflammatory bowel diseases (IBD).

Keywords: pituitary adenylate cyclase-activating polypeptide (PACAP), subacute ileitis, Th1-type immunopathology, human fecal microbiota transplantation, gut-brain axis, preclinical intervention study

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INTRODUCTION

The Pituitary adenylate cyclase-activating polypeptide (PACAP) could be first identified in the hypothalamus exerting adenylate cyclase stimulating activity within the pituitary gland (1). Being part of the vasoactive intestinal peptide (VIP)/secretin/glucagon family, the neuropeptide shares 68% sequence homology with VIP and presents with two biologically active amidated forms (i.e., PACAP 27 and PACAP38) after alternative splicing from its pre-pro precursor (1, 2). Beyond the nervous system, PACAP expression can be found in many peripheral organs within the reproductive, respiratory, endocrine and digestive system as well as in lymphoid compartments including immune cells (2). PACAP is able to bind to VPAC1, VPAC2, and PAC1 receptors on innate immune cells including macrophages and lymphocytes (3–5). Given its virtual ubiquitous expression, PACAP presents with a variety of cyto-protective properties including anti-inflammatory and anti-apoptotic effects (5, 6). In experimental models of encephalomyelitis and arthritis, for instance, distinct anti-inflammatory effects following exogenous PACAP application have been demonstrated (7, 8). Data regarding inflammation-ameliorating properties of synthetic PACAP in the gastrointestinal tract are limited, however. $PACAP^{-/-}$ mice suffered from more severe acute colitis following dextran sodium sulfate (DSS) challenge as compared to wildtype counterparts (9, 10).

Human inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis comprise chronic inflammatory conditions with acute episodes within the gastrointestinal tract and are of multi-factorial etiology (11–13). Most *in vivo* studies mimicking human IBD have applied experimental models of the large intestines so far, whereas, however, small intestinal inflammation models are rather scarce (14).

In our previous studies we applied an acute ileitis model characterized by a severe T cell-driven immunopathology with a lethal outcome within 1 week after peroral high-dose (i.e., >50 cysts) *Toxoplasma gondii* infection of mice (14–17). This high-dose *T. gondii* infection model mimics key features of the acute phase of human Crohn's disease ("ileitis terminalis"), given (i) the predilection site of the terminal ileum, (ii) the underlying T helper cell (Th)−1 immunopathology that is (iii) associated with marked shifts in gut microbiota composition (dysbiosis) toward Gram-negative gut commensals, (iv) further contributing to an acceleration of the inflammatory scenario via Toll-like receptor (TLR)−4 dependent signaling of lipopolysaccharide (LPS) derived from the Gram-negative commensal bacterial cell walls (14). We were able to show that treatment of mice with synthetic PACAP could efficiently ameliorate acute ileitis and even extra-intestinal sequelae of *T. gondii* infection in a time-of-treatment dependent fashion with highest efficacy during a prophylactic regimen when starting PACAP application prior ileitis induction (17). The within 1 week lethal outcome of the hyper-acute inflammatory scenario following high-dose *T. gondii* infection needs to be considered as a limitation of the applied gut inflammation model, however.

This prompted us to unravel potential immune-modulatory properties of PACAP during small intestinal inflammation of less acute severity. Since the host specific gut microbiota is known to be essentially involved in the onset, progress, and outcome of distinct immunopathological conditions including intestinal inflammation (15, 18, 19), we generated (with respect to their gut microbiota) "humanized" mice. Very recently we were able to show that within 9 days following peroral infection with a low-dose (i.e., 1 cyst) of *T. gondii*, mice harboring a human gut microbiota develop non-lethal subacute ileitis characterized by increased T cell-dependent gut epithelial apoptosis and pro-inflammatory cytokine secretion in intestinal and extra-intestinal compartments (20). Furthermore, low-dose *T. gondii* infected mice displayed rather mild-to-moderate histopathological changes of the ileal mucosa and lamina propria, whereas no transmural small intestinal necrosis like in the lethal high-dose infection model could be observed. In the present preclinical intervention study we assessed whether therapeutic PACAP application starting 3 days after ileitis induction (i) resulted in disease-alleviating effects in the intestinal tract, (ii) was associated with distinct shifts in gut microbiota composition, and furthermore, (iii) whether potential PACAP-induced anti-inflammatory effect could also be observed in extra-intestinal organs or (iv) even in systemic compartments.

MATERIALS AND METHODS

Generation of Mice With a Human Gut Microbiota by Fecal Microbiota Transplantation

Female C57BL/6j mice were raised and maintained under specific pathogen-free (SPF) conditions in the Forschungseinrichtungen für Experimentelle Medizin (FEM, Charité - University Medicine, Berlin, Germany). Mice with a depleted microbiota (i.e., secondary abiotic mice) were generated as reported earlier (15, 18). Briefly, eight-week-old mice were kept in autoclaved cages and treated with an antibiotic cocktail for 8 weeks containing ampicillin plus sulbactam (1 g/L; Ratiopharm, Germany), vancomycin (500 mg/L; Cell Pharm, Germany), ciprofloxacin (200 mg/L; Bayer Vital, Germany), imipenem (250 mg/L; MSD, Germany) and metronidazole (1 g/L; Fresenius, Germany) (*ad libitum*). Successful depletion of the gut microbiota was confirmed in fecal samples by both, culture and molecular (16S rRNA based) methods as stated elsewhere (18, 21). In order to guarantee antibiotic washout, the antibiotic cocktail was replaced by sterile tap water (*ad libitum*) 3 days before human fecal microbiota transplantation (FMT). Fresh fecal samples that were negative for enteropathogenic bacteria, viruses, and parasites were donated from five healthy human individuals, dissolved in sterile phosphate buffered saline (PBS; Gibco, Life Technologies, United Kingdom) and stored

Abbreviations: CFU, colony forming units; DSS, dextran sodium sulfate; FMT, fecal microbiota transplantation; H&E, hematoxylin and eosin; Hma, human microbiota associated; HPF, high power field; IBD, inflammatory bowel disease; IFN, interferon; IL, interleukin; i.p., intraperitoneal; MLN, mesenteric lymph nodes; PACAP, Pituitary adenylate cyclase-activating polypeptide; PBS, phosphate buffered saline; p.i., post-infection; PLC, placebo; qRT-PCR, quantitative real-time polymerase chain reaction; SPF, specific pathogen free; Th, T helper cell; TNF, tumor necrosis factor; VIP, vasoactive intestinal peptide.

at −80°C until usage as described previously (18). Immediately before FMT, individual fecal aliquots were thawed, pooled and applied to secondary abiotic mice by gavage on three consecutive days (18). Between individual FMT challenges, bacterial loads varied <0.5 orders of magnitude (**Figure S1**). For appropriate establishment of the complex human gut microbiota within the murine host, mice were kept for 12 days before subacute ileitis induction. Immediately before peroral *T. gondii* infection (d0) and at the end of the observation period [i.e., day 9 post-infection (p.i.)] individual fecal samples were collected from human microbiota associated (hma) mice for subsequent quantification of the main gut bacterial groups by molecular methods as described elsewhere (15, 18, 19).

Induction of Subacute Ileitis, Determination of Parasitic Loads

For induction of subacute ileitis, hma mice were perorally challenged with one cyst of *T. gondii* strain ME49 in 0.3 mL brain suspension by gavage as reported earlier (20). *T gondii* DNA was quantitated in ileal *ex vivo* biopsies as stated previously (16).

Treatment

PACAP38 was synthesized at the Department of Medical Chemistry, University of Szeged (Hungary) and applied to mice with a daily dose of 1.5 mg per kg body weight (in PBS) (9, 17). Mice received either the synthetic PACAP38 or PBS as placebo control (PLC) via the intraperitoneal (i.p.) route (0.3 mL) from day 3 p.i. until day 8 p.i. once daily.

Clinical Conditions and Sampling Procedures

Clinical conditions of mice including body weight loss were monitored daily. Nine days post ileitis induction mice were sacrificed by isoflurane treatment (Abbott, Germany). Cardiac blood and *ex vivo* biopsies were derived from mesenteric lymph nodes (MLN), lung, liver, ileum and colon under aseptic conditions. Respective tissue samples were taken from each mouse in parallel for immunological, immunohistochemical, and microbiological analyses. Whereas the small intestinal lengths were assessed by measuring the distance from the duodenum leaving the stomach to the ileal-cecal valve, the colonic lengths were measured from the ileal-cecal valve to the rectum with a ruler and expressed in cm.

Histopathology and Immunohistochemistry

Ex vivo biopsies were derived from the terminal ileum, colon, lung, and liver, fixed in 5% formalin and embedded in paraffin. Histopathological changes were quantitated in 5 µm thin, with hematoxylin and eosin (H&E) stained ileal paraffin sections applying a standardized histopathological scoring system ranging from 0 to 6 as stated elsewhere (15).

Paraffin sections (5 µm) were further analyzed applying *in situ* immunohistochemistry as reported previously (22). Briefly, in order to quantitate apoptotic cells and T lymphocytes, primary antibodies against cleaved caspase-3 (Asp175, #9661, Cell Signaling, Leiden, Netherlands; 1:200), and CD3 (#IR50361-2, Dako, Santa Clara, CA, USA; 1:5) were applied, respectively. The average number of positively stained cells (within at least six high power fields (HPF), 0.287 mm^2, 400x magnification) was assessed by an independent and blinded investigator.

Pro-inflammatory Cytokine Secretion

Ileal and colonic tissue samples (∼1 cm^2) were cut longitudinally and washed in PBS. Respective intestinal *ex vivo* biopsies as well as samples derived from MLN (3 lymph nodes), liver samples (∼1 cm^2) and lung were placed in 24-flat-bottom well-culture plates (Falcon, Germany) supplemented with 500 µL serum-free RPMI 1640 medium (Gibco, life technologies), penicillin (100 U/mL, Biochrom, Germany) and streptomycin (100 µg/mL; Biochrom). After overnight incubation at 37°C, culture supernatants were taken and tested for IL-6, TNF, and IFN-γ secretion applying the Mouse Inflammation Cytometric Bead Assay (CBA; BD Bioscience) on a BD FACSCanto II flow cytometer (BD Bioscience). Systemic pro-inflammatory cytokine concentrations were measured in serum samples.

Molecular Analysis of the Human Fecal Donor Suspensions and the Intestinal Microbiota

Human fecal donor suspensions as well as fresh ileal and colonic luminal samples were immediately transferred to liquid nitrogen and stored at −80°C until further analyses. Fecal DNA extraction was performed as reported earlier (15). Briefly, the amount of DNA was assessed with a Quant-iT PicoGreen reagent (Invitrogen, UK) and adjusted to 1 ng per µL. The main human gut bacterial groups including enterobacteria, enterococci, lactobacilli, bifidobacteria, *Bacteroides/Prevotella* species, *Clostridium coccoides* group, and *Clostridium leptum* group as well as the total eubacterial loads were determined applying quantitative real-time polymerase chain reaction (qRT-PCR) and species-, genera- or group-specific 16S rRNA gene primers (Tib MolBiol, Germany) as indicated (**Figure S2**) and further described previously (19, 23) (expressed as 16S rRNA gene copies per ng DNA).

Bacterial Translocation

In order to survey viable bacteria translocating from the gastrointestinal tract to extra-intestinal including systemic tissue sites, *ex vivo* biopsies were taken from MLN, lungs, and liver, homogenized in sterile PBS and analyzed in serial dilutions on defined solid culture media as reported previously (15, 24). Cardiac blood was incubated in thioglycolate enrichment broths (BD Bioscience, Germany) for at least 7 days at 37°C and then streaked on culture media (25, 26). For at least 2 days bacteria were grown under aerobic, microaerobic and anaerobic conditions at 37°C.

Statistical Analysis

Medians and levels of significance were determined by the one-way ANOVA test followed by Tukey post-correction test for multiple comparisons. Two-sided probability (p) values ≤0.05 were considered significant. Experiments were reproduced three times.

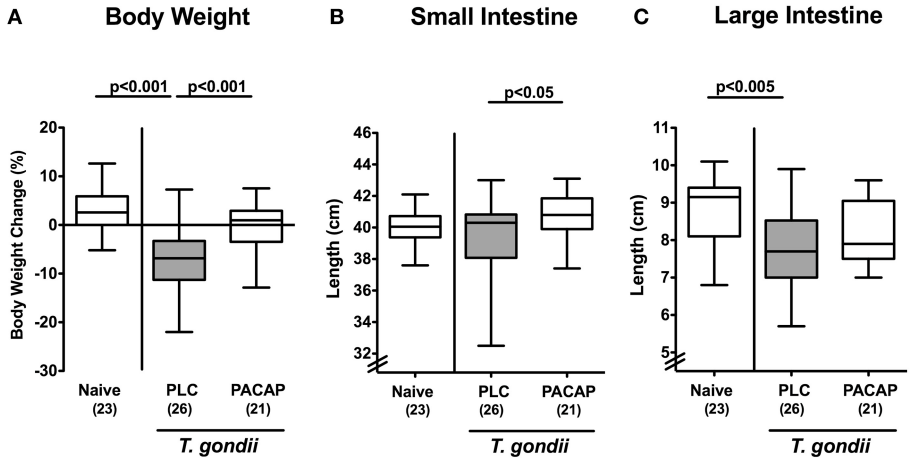

FIGURE 1 | Macroscopic effects in PACAP treated mice with a human gut microbiota suffering from subacute ileitis. Subacute ileitis was induced by *T. gondii* infection of mice harboring a human gut microbiota (day 0). Starting 3 days post-infection (p.i.), mice were either treated with PACAP or placebo (PLC). Uninfected mice with a human microbiota served as control animals (Naive). At day 9 p.i., **(A)** relative body weight loss, **(B)** small intestinal, and **(C)** large intestinal lengths were assessed. Box plots represent the 75 and 25th percentiles of the median (black bar inside the boxes). The total range and significance levels determined by one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are shown. Total numbers of analyzed animals are given in parentheses. Data were pooled from four independent experiments.

RESULTS

Macroscopic Sequelae in PACAP Treated Mice With a Human Gut Microbiota Suffering From Subacute Ileitis

In the present preclinical intervention study we addressed whether therapeutic application of synthetic PACAP dampened pro-inflammatory responses in intestinal and extra-intestinal including systemic compartments of mice with a human gut microbiota suffering from subacute ileitis. The small intestinal immunopathology was induced by peroral low dose *T. gondii* infection on day 0 (20). Three days post ileitis induction, hma mice were treated with synthetic PACAP for 6 days in total and clinical conditions including the body weights were monitored. Remarkably, *T. gondii* infected placebo (PLC) control mice exhibited substantial body weight loss until day 9 p.i., whereas this was not the case in PACAP treated mice (**Figure 1A**). Given that intestinal inflammation is accompanied by a significant shortening of the inflamed intestine (15, 18, 27), we measured small intestinal lengths upon necropsy. At day 9 p.i., PACAP treated mice displayed slightly longer small intestines as compared to PLC control animals (**Figure 1B**). Of note, the standard deviation within the PLC group was relatively high, which might explain the non-significant differences compared to the naive cohort (**Figure 1B**). Even though the terminal ileum is well-known to be the predilection site of *T. gondii* induced immunopathology (14), we also assessed the lengths of the large intestines. Interestingly, PLC, but not PACAP treated mice displayed significantly shorter large intestines at day 9 p.i. as compared to naive mice (**Figure 1C**), pointing toward inflammatory responses beyond the terminal small intestine, additionally affecting the large intestinal tract. Hence, PACAP treatment resulted in a better clinical / macroscopic outcome of *T. gondii* induced subacute ileitis. To rule out that the observed differences in disease outcomes might be due to different *T. gondii* infection efficiencies, we assessed parasitic DNA concentrations in ileal *ex vivo* biopsies at day 9 p.i., but detected comparable ileal *T. gondii* loads in mice of the PACAP and PLC cohort (not shown).

Intestinal Anti-inflammatory Effects in PACAP Treated Mice With a Human Gut Microbiota Suffering From Subacute Ileitis

We next addressed PACAP-induced anti-inflammatory effects in the intestinal tract on microscopic level. At day 9 following ileitis induction ileal histopathological scores were lower in PACAP treated hma mice as compared to PLC control animals (**Figure 2**). Of note, we could not observe significant differences in histopathological changes in the mucosa and lamina propria of H&E stained colonic paraffin sections (not shown). Since apoptosis is regarded a reliable parameter for the histopathological grading of intestinal inflammation (16), we further assessed *T. gondii* induced apoptotic cell responses in the intestines by quantification of caspase3+ intestinal epithelial cells applying *in situ* immunohistochemistry. At day 9 p.i., hma mice displayed multifold increased numbers of apoptotic ileal epithelial cells (**Figure 3A**). These increases were, however, less pronounced in PACAP treated mice (**Figure 3A**; **Figure S3A**).

Given that T lymphocytes are the major driving forces of *T. gondii* induced ileitis (14), we additionally stained ileal paraffin sections with CD3 antibodies. Ileitis induction was, in fact, accompanied by a marked increase in CD3+ ileal epithelial cell numbers until day 9 p.i. (**Figure 3B**, **Figure S3B**), but to a

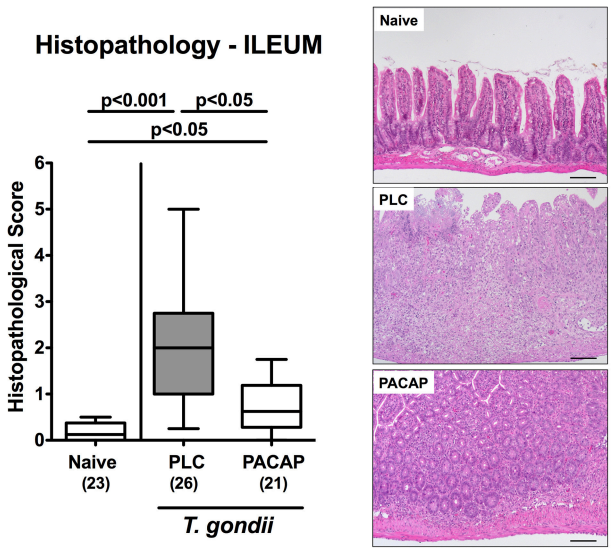

FIGURE 2 | Microscopic effects in PACAP treated mice with a human gut microbiota suffering from subacute ileitis. Subacute ileitis was induced by *T. gondii* infection of mice harboring a human gut microbiota (day 0). Starting 3 days post-infection (p.i.), mice were either treated with PACAP or placebo (PLC). Uninfected mice with a human microbiota served as control animals (Naive). At day 9 p.i., histopathological changes within the ileum were quantitated in H&E stained ileal paraffin sections applying a standardized histopathological scoring system (left). Box plots represent the 75 and 25th percentiles of the median (black bar inside the boxes). The total range and significance levels determined by one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are shown. Total numbers of analyzed animals are given in parentheses. Data were pooled from four independent experiments. Histopathological changes are illustrated in photomicrographs representative for four independent experiments (right; 100x magnification, scale bar 100 μm).

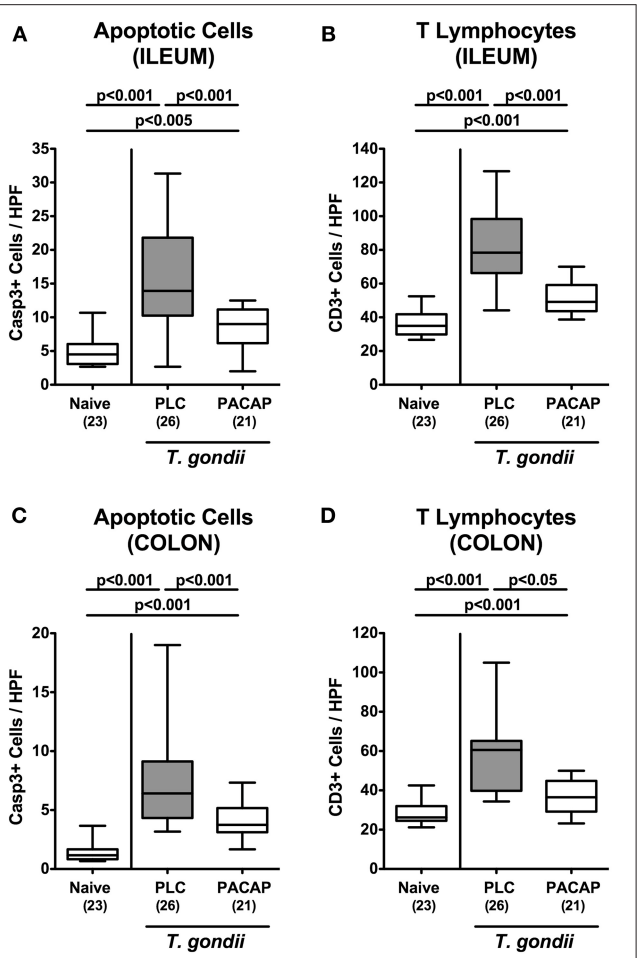

FIGURE 3 | Apoptotic epithelial cell and T lymphocyte responses in the intestinal tract following PACAP treatment of mice with a human gut microbiota suffering from subacute ileitis. Subacute ileitis was induced by *T. gondii* infection of mice harboring a human gut microbiota (day 0). Starting 3 days post-infection (p.i.), mice were either treated with PACAP or placebo (PLC). Uninfected mice with a human microbiota served as control animals (Naive). At day 9 p.i., the average numbers of apoptotic epithelial cells [Casp3+; **(A,C)**] and of T lymphocytes [CD3+; **(B,D)**] in at least six high power fields (HPF) were quantitatively assessed in ileal **(A,B)** and colonic **(C,D)** paraffin sections applying *in situ* immunhistochemistry. Box plots represent the 75 and 25th percentiles of the median (black bar inside the boxes). The total range and significance levels determined by one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are shown. Total numbers of analyzed animals are given in parentheses. Data were pooled from four independent experiments.

significantly lesser extent upon PACAP treatment (**Figure 3B**, **Figure S3B**). Remarkably, *T. gondii*-induced increases in both, caspase3+ and CD3+ cells could also be observed in the epithelia and mucosa / lamina propria of the large intestines, respectively (**Figures 3C,D, Figures S3C,D**). Like in the ileal compartment, PACAP treatment was accompanied with significantly less distinct apoptosis and abundance of T lymphocytes in the large intestinal tract (**Figures 3C,D, Figures S3C,D**).

We further assessed pro-inflammatory cytokine secretion in intestinal *ex vivo* biopsies. At day 9 p.i., PLC, but not PACAP treated mice exhibited higher IL-6 concentrations in their ileum as compared to naive counterparts (**Figure 4A**). In addition, TNF secretion was far less pronounced in the ileum and MLN of mice from the PACAP cohort as compared to PLC control animals (**Figures 4B,C**). Hence, PACAP exerts potent inflammation-alleviating effects in the intestinal tract of hma mice during subacute ileitis that are not restricted to the terminal ileum.

Changes in Gut Microbiota Composition in PACAP Treated Mice With a Human Gut Microbiota Suffering From Subacute Ileitis

Given that intestinal inflammatory conditions are accompanied by shifts in commensal gut microbiota composition of mice and men termed dysbiosis (15, 19, 26, 28–30), we quantitatively surveyed the main gut bacterial groups during subacute ileitis development in PACAP and PLC treated mice applying culture-independent 16S rRNA based methods (**Figures 5A-H**). Irrespective of the treatment regimen, the total eubacterial loads in the ileal lumen slightly declined until day 9 p.i. (**Figure 5A**). Conversely, ileitis development was accompanied by higher gene numbers of enterobacteria and enterococci in the ilea of PLC, but not PACAP mice (**Figures 5B,C**), whereas lactobacilli loads were higher in PACAP treated mice at day 9 p.i. as compared to both,

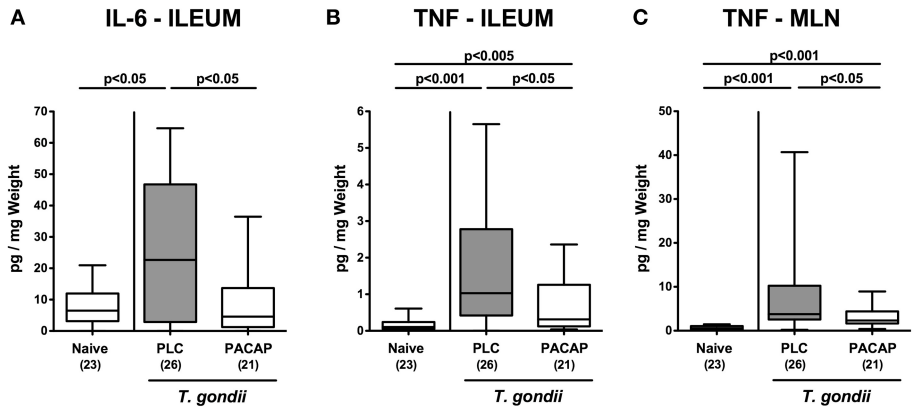

FIGURE 4 | Intestinal pro-inflammatory cytokine secretion upon PACAP treatment of mice with a human gut microbiota suffering from subacute ileitis. Subacute ileitis was induced by *T. gondii* infection of mice harboring a human gut microbiota (day 0). Starting 3 days post-infection (p.i.), mice were either treated with PACAP or placebo (PLC). Uninfected mice with a human microbiota served as control animals (Naive). At day 9 p.i., secretion of pro-inflammatory cytokines such as IL-6 **(A)** and TNF **(B,C)** were assessed in *ex vivo* biopsies derived from the ileum **(A,B)** and from mesenteric lymph nodes [MLN; **(C)**]. Box plots represent the 75 and 25th percentiles of the median (black bar inside the boxes). The total range and significance levels determined by one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are shown. Total numbers of analyzed animals are given in parentheses. Data were pooled from four independent experiments.

T. gondii infected PLC treated mice and naive control animals (**Figure 5D**). Remarkably, bifidobacteria were only marginally abundant in mice suffering from subacute ileitis (**Figure 5E**), with a trend toward higher loads in PACAP vs. PLC mice at day 9 p.i. (**Figure 5E**). Furthermore, *Clostridium coccoides* gene numbers were lower in the ileum derived from *T. gondii* infected mice of either cohort (**Figure 5G**), whereas this was the case for *Clostridium leptum* in PLC mice (**Figure 5H**), but not PACAP treated counterparts (**Figure 5H**). Hence, subacute ileitis development in hma mice was accompanied with distinct shifts in the microbiota composition of the inflamed ileum, but to a lesser extent upon PACAP treatment.

Extra-intestinal Anti-inflammatory Effects in PACAP Treated Mice With a Human Gut Microbiota Suffering From Subacute Ileitis

We next addressed whether anti-inflammatory effects upon PACAP treatment of hma mice with subacute ileitis were restricted to the intestinal tract or might also be observed in extra-intestinal including systemic compartments. Nine days following ileitis induction multi-fold increased numbers of apoptotic cell numbers could be observed in the livers derived from PLC and PACAP treated mice as compared to naive animals (**Figure 6A**; **Figure S4A**), but with lower counts in the latter as compared to the former (**Figure 6A**; **Figure S4A**). Irrespective of the treatment regimen, increases in apoptotic hepatic cell numbers were paralleled by more than three-fold higher numbers of T lymphocytes in the livers of *T. gondii* infected as compared to naive mice (**Figure 6B**; **Figure S4B**). Furthermore, multi-fold elevated numbers of both apoptotic cells and T lymphocytes could be assessed in the lungs during subacute ileitis (**Figures 6C,D**; **Figures S4C,D**), but with lower counts in PACAP as compared to PLC treated mice at day 9 p.i. (**Figures 6C,D**; **Figures S4C,D**).

We next measured pro-inflammatory cytokine secretion in *ex vivo* biopsies derived from respective extra-intestinal compartments and detected less distinctly increased INF-γ concentrations in the liver and lungs of PACAP as compared to PLC treated mice at day 9 p.i. (**Figure 7**). As for the ileum, subacute ileitis induction was further accompanied by elevated systemic concentrations of pro-inflammatory cytokines such as TNF and IL-6 (**Figure 8**). Strikingly, PACAP treatment resulted in ∼50% lower TNF and IL-6 concentrations measured in serum samples taken at day 9 p.i. as compared to PLC control mice (**Figure 8**). Hence, the profound anti-inflammatory effects exerted by PACAP treatment of hma mice during subacute ileitis were not restricted to the intestinal tract, but could also be observed in extra-intestinal and even systemic compartments.

DISCUSSION

Several *in vitro* and *in vivo* studies revealed that PACAP exerts its neuroprotective properties via immune-modulatory and anti-apoptotic mechanisms (5, 6). Given its virtual ubiquitous expression, however, PACAP rather acts as a pleiotropic immune regulator and hence, also beyond the nervous system (6, 31). In fact, our previous work revealed that synthetic PACAP application starting prior acute ileitis induction (i.e., prophylactic regimen) ameliorated intestinal as well as extra-intestinal sequelae of peroral high-dose *T. gondii* infection in a time-of-treatment dependent manner (17) that is characterized by a T cell-driven pro-inflammatory cytokine storm with fatal outcome within 1 week (14, 15). In the present preclinical intervention study we provide additional insights into the inflammation-ameliorating properties of exogenous PACAP. The here applied subacute infection model following peroral low-dose *T. gondii* infection of mice with a human gut microbiota has been established by our group very recently (20) and is

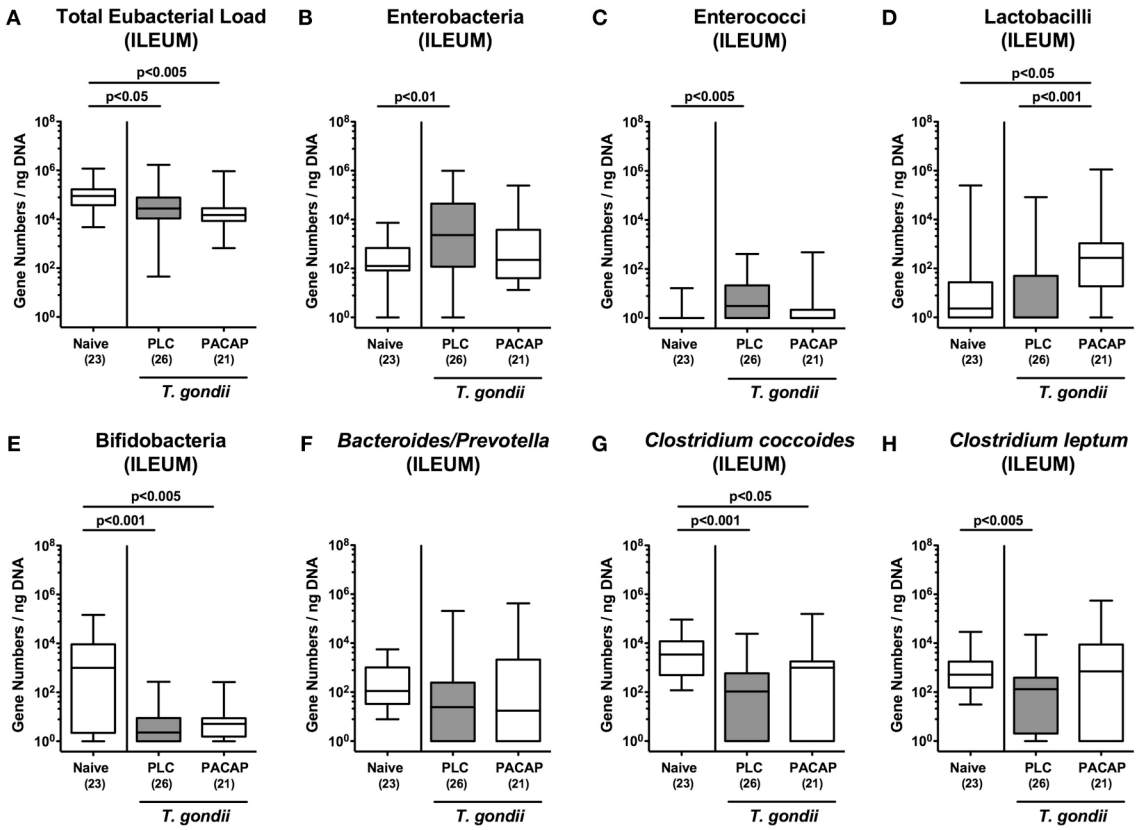

FIGURE 5 | Colonic microbiota changes following PACAP treatment of mice with a human gut microbiota suffering from subacute ileitis. Subacute ileitis was induced by *T. gondii* infection of mice harboring a human gut microbiota (day 0). Starting 3 days post-infection (p.i.), mice were either treated with PACAP or placebo (PLC). Uninfected mice with a human microbiota served as control animals (Naive). At day 9 p.i., the microbiota composition of the ileal lumen **(A-H)** was determined by quantitative Real-Time PCR amplifying bacterial 16S rRNA variable regions of the main intestinal bacterial groups (expressed as 16S rRNA gene numbers per ng DNA) including the total eubacterial load as indicated. Box plots represent the 75 and 25th percentiles of the median (black bar inside the boxes). The total range and significance levels determined by one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are shown. Total numbers of analyzed animals are given in parentheses. Data were pooled from four independent experiments.

characterized by a non-lethal, far less acute course of small intestinal inflammation (as compared to high-dose *T. gondii* infection), and importantly, mimics human gut microbiota conditions. The low-dose *T. gondii* infection model of hma mice thus provides valuable opportunities to further dissect the molecular mechanisms underlying the interactions between pathogen, host immunity, and human gut microbiota during small intestinal inflammation (20).

In our present work we demonstrate that PACAP applied in a therapeutic regimen (starting post ileitis induction) exerts anti-inflammatory effects during subacute ileitis of (with respect to their gut microbiota) "humanized" mice as indicated by (1) better clinical / macroscopic conditions (including lack of body weight loss and of shrinkage of the intestinal lengths during ileitis development), (2) less distinct histopathological changes in the terminal ileum, (3) lower numbers of apoptotic epithelial cells and of T lymphocytes in both, ileum and colon, (4) less intestinal secretion of pro-inflammatory cytokines such as TNF and IL-6, (5) less apoptosis in extra-intestinal organs such as liver and lungs that is accompanied by (6) lower pulmonal T cell numbers, and (7) less IFN-γ secretion in liver and lungs, and, remarkably, (8) lower systemic concentrations of pro-inflammatory cytokines such as TNF and IL-6. Furthermore, (9) inflammation-associated gut microbiota shifts were less pronounced following PACAP as compared to PLC treatment.

The potent anti-inflammatory effects of exogenous PACAP within the intestinal tract as assessed in our actual and previous (17) study are further supported by two previous reports demonstrating that PACAP$^{-/-}$ mice were suffering from more severe DSS-induced colitis as compared to wildtype counterparts (9, 10). When synthetic PACAP was applied via the intraperitoneal route, however, the inflammatory phenotype could be alleviated (9).

Even though peroral *T. gondii* infection of susceptible mice is known to primarily affect the terminal ileum, we extended our focus to the large intestines here. We did indeed observe marked *T. gondii* induced colonic inflammatory responses as indicated by multi-fold increased numbers of apoptotic

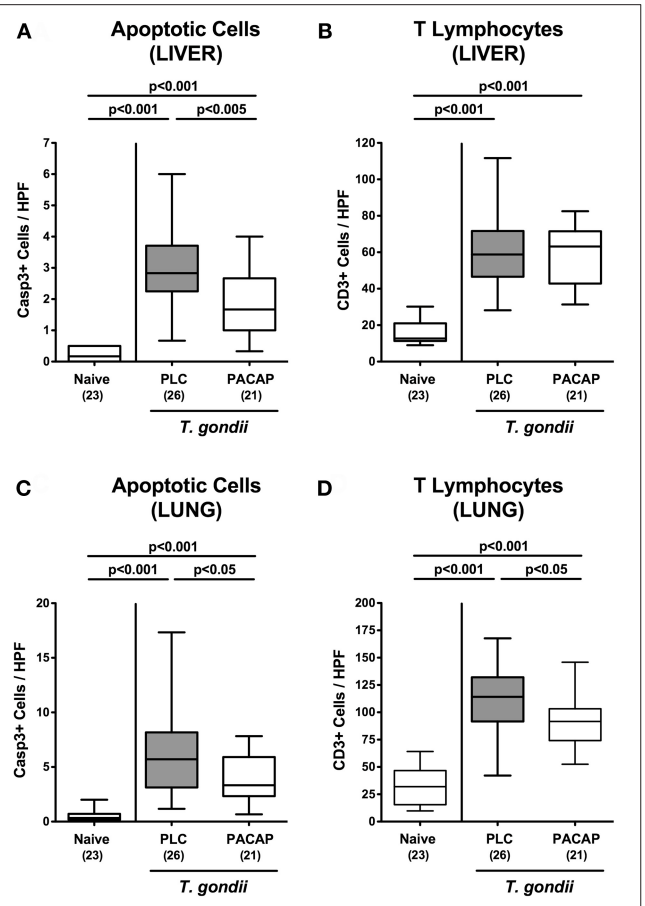

FIGURE 6 | Apoptotic epithelial cell and T lymphocyte responses in extra-intestinal compartments following PACAP treatment of mice with a human gut microbiota suffering from subacute ileitis. Subacute ileitis was induced by *T. gondii* infection of mice harboring a human gut microbiota (day 0). Starting 3 days post-infection (p.i.), mice were either treated with PACAP or placebo (PLC). Uninfected mice with a human microbiota served as control animals (Naive). At day 9 p.i., the average numbers of apoptotic epithelial cells [Casp3+; **(A,C)]** and of T lymphocytes [CD3+; **(B,D)**] in at least six high power fields (HPF) were quantitatively assessed in paraffin sections of *ex vivo* biopsies derived from liver **(A,B)** and lung **(C,D)** applying *in situ* immunhistochemistry. Box plots represent the 75 and 25th percentiles of the median (black bar inside the boxes). The total range and significance levels determined by one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are shown. Total numbers of analyzed animals are given in parentheses. Data were pooled from four independent experiments.

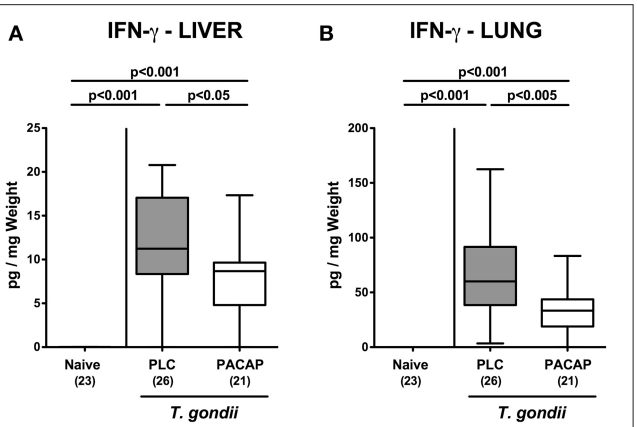

FIGURE 7 | Extra-intestinal pro-inflammatory cytokine responses following PACAP treatment of mice with a human gut microbiota suffering from subacute ileitis. Subacute ileitis was induced by *T. gondii* infection of mice harboring a human gut microbiota (day 0). Starting 3 days post-infection (p.i.), mice were either treated with PACAP or placebo (PLC). Uninfected mice with a human microbiota served as control animals (Naive). At day 9 p.i., IFN-γ secretion was assessed in *ex vivo* biopsies derived from the liver **(A)** and from lung **(B)**. Box plots represent the 75 and 25th percentiles of the median (black bar inside the boxes). The total range and significance levels determined by one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are shown. Total numbers of analyzed animals are given in parentheses. Data were pooled from four independent experiments.

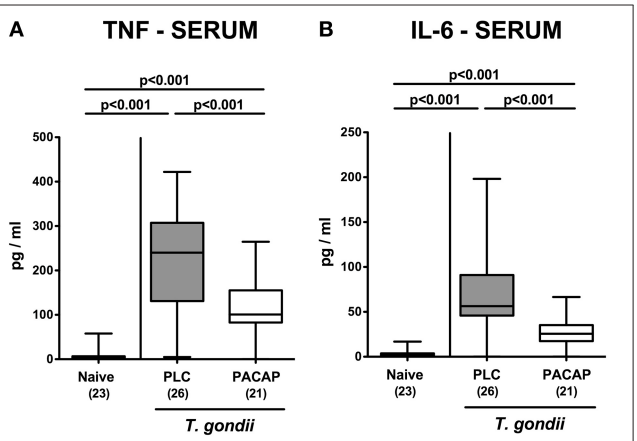

FIGURE 8 | Systemic pro-inflammatory cytokine responses following PACAP treatment of mice with a human gut microbiota suffering from subacute ileitis. Subacute ileitis was induced by *T. gondii* infection of mice harboring a human gut microbiota (day 0). Starting 3 days post-infection (p.i.), mice were either treated with PACAP or placebo (PLC). Uninfected mice with a human microbiota served as control animals (Naive). At day 9 p.i., secretion of pro-inflammatory cytokines such as TNF **(A)** and IL-6 **(B)** were measured in serum samples. Box plots represent the 75 and 25th percentiles of the median (black bar inside the boxes). The total range and significance levels determined by one-way ANOVA test followed by Tukey post-correction test for multiple comparisons are shown. Total numbers of analyzed animals are given in parentheses. Data were pooled from four independent experiments.

colonic epithelial cells that could be effectively lowered by therapeutic PACAP application, which also held true for T lymphocytes within the colonic mucosa and lamina propria. In previous *in vitro* studies PACAP was shown to inhibit proliferation and migration of T lymphocytes and associated pro-inflammatory cytokine release (3, 32). An *in vivo* study further revealed that PACAP treated mice suffering from acute peritonitis displayed a diminished influx of lymphocytes into the peritoneal cavity (33).

Interestingly, synthetic PACAP was not sufficient to lower *T. gondii* induced colonic pro-inflammatory cytokine secretion (not shown), whereas this was the case in *ex vivo* biopsies derived from the ileum and from MLN. Our present observations are supported by our previous reports in both acute (25, 26) and subacute (20) murine ileitis further emphasizing that *T.*

gondii induced immunopathology does not exclusively affect the terminal ileum, but might also affect the large intestines.

The well-known anti-apoptotic properties of PACAP were not restricted to the intestinal tract, but could also be assessed in extra-intestinal organs such as the liver and lungs and were paralleled by decreased IFN-γ secretion in respective organs. In line, PACAP could exert potent protective effects in human hepatocytes *in vitro* and in a murine model of liver ischemia and oxidative stress (34, 35) as well as in endotoxin-induced acute lung inflammation (36). Notably, synthetic PACAP analogs have been developed for the treatment of bronchial asthma given the anti-inflammatory and broncho-relaxant properties of the neuropeptide (36, 37).

We further addressed whether the observed inflammation-alleviating responses in PACAP treated mice were paralleled by less translocation of viable bacteria originating from the commensal gut microbiota to extra-intestinal including systemic compartments due to less distinct epithelial barrier damage. It is well-known that during acute ileitis bacterial commensals including *Escherichia coli* overgrowing the ileal lumen might migrate through the compromised intestinal epithelial barrier (i.e., "leaky gut"), come in close contact to immune cells residing in the lamina propria which might subsequently result in an exacerbation of the inflammatory immune responses (38–40). Interestingly, in our actual study bacteria could neither be cultured from MLN, nor from extra-intestinal compartments including liver, lungs and cardiac blood. Our recent study, however, revealed that mean commensal bacterial translocation rates of more than 80% could be assessed in liver and lungs during lethal acute ileitis (25, 26).

One might argue that the better outcome observed in PACAP treated hma mice with *T. gondii* induced ileitis was due to direct anti-microbial effects of the applied synthetic compound, given that both anti-parasitic (directed against *Trypanosoma brucei*) (41) and anti-bacterial (42) effects have been reported recently. In both our previous and actual studies we could, however, exclude any anti-microbial effects of the working solutions containing synthetic PACAP *in vitro* (17). Furthermore, the start of exogenous PACAP 3 days after *T. gondii* infection, in addition to comparable parasitic DNA loads as assessed in ileal *ex vivo* biopsies argue against a biological relevant anti-microbial effect of PACAP.

Meanwhile it is well-established that the orchestrated mutualistic microbiota-host relationships are of utmost importance for host cell physiology, immune homeostasis and resistance to disease (21, 43). Perturbations within the complex microbial ecosystem in the gastrointestinal tract are associated with increased susceptibility of the host to distinct intestinal morbidities including IBD, irritable bowel syndrome and coeliac disease (21, 44, 45). Likewise, intestinal inflammatory conditions are paralleled by shifts in the intestinal microbiota composition (15, 25, 26, 28–30, 46, 47). This phenomenon could also be observed in our actual survey of the microbiota composition within the inflamed ileal lumen, given that subacute ileitis development was associated with increases in enterobacteria (including *E. coli*) and enterococci, whereas obligate anaerobic *Clostridium coccoides* and *Clostridium leptum* gene numbers decreased until day 9 p.i. These microbiota shifts are supported by our previous results obtained from lethal acute ileitis of mice, but with a conventional (i.e., murine) gut microbiota (15, 46, 47). Remarkably, neither shifts toward increased enterobacteria and enterococci, nor to decreased numbers of *Clostridium leptum* could be assessed in the ilea of PACAP treated mice. Furthermore, PACAP treatment was associated with higher abundances of (potentially probiotic) lactobacilli that had been reduced during acute ileitis (15).

Interestingly, PACAP could inhibit TLR-4 activation in a model of traumatic brain injury (48). Given that *T. gondii* induced ileitis is initiated and further worsened by TLR-4 dependent signaling of bacterial LPS originating from the cell walls of Gram-negative commensals including enterobacteria such as *E. coli* accumulating in the inflamed ileal lumen (46, 47), alleviation of the TLR-4 dependent scenario constitutes a mechanistic corner stone of the multi-facetted "health-beneficial actions" of PACAP treatment.

Strikingly, exogenous PACAP could not only sufficiently dampen ileal, but also serum TNF and IL-6 concentrations, pointing toward potent systemic anti-inflammatory properties of the synthetic compound, which could also be observed in lethal acute ileitis (17). These systemic effects of exogenous PACAP are supported by several studies where PACAP could efficiently prevent from experimental endotoxin sepsis and shock (49–51).

Given the time-of-treatment dependent anti-inflammatory effect of exogenous PACAP observed during acute ileitis (17), one might further argue that starting PACAP application to hma mice before subacute ileitis induction (i.e., prophylactic regimen) might yield even more pronounced anti-apoptotic and anti-inflammatory effects in intestinal and extra-intestinal including systemic compartments. This should be addressed in future studies.

In summary, our preclinical intervention study provides strong evidence that synthetic PACAP alleviates subacute ileitis and extra-intestinal including systemic sequelae of T cell-driven immunopathology. We conclude that synthetic PACAP might open novel options for the (adjunct) therapy and/or prophylaxis of intestinal inflammation including IBD and further supports the pathophysiological role of the gut-brain axis.

ETHICS STATEMENT

After approval of the protocols by the commission for animal experiments headed by the "Landesamt für Gesundheit und Soziales" (LaGeSo, Berlin; registration numbers G0368/11 and G0039/15), the mouse experiments were performed according to the European Guidelines for animal welfare (2010/63/EU). Animal welfare was monitored daily by assessment of clinical conditions and weight loss of mice.

Mice with body weight loss of more than 20% were euthanized by cervical dislocation in accordance with the guidelines of the local authorities.

AUTHOR CONTRIBUTIONS

SB provided advice in design and performance of experiments, co-wrote paper. UE and AG performed experiments, analyzed data, co-edited paper. AK analyzed data, co-edited paper. ID, AT, and DR suggested critical parameters in design of experiments, co-edited paper. MH designed and performed experiments, analyzed data, wrote paper.

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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.2019.00554/full#supplementary-material

Figure S1 | Microbiota composition of human donor feces. Before human FMT of secondary abiotic mice on 3 consecutive days, the main gut bacterial groups were quantitated in human fecal donor suspensions. Applying quantitative RT-PCR analysis, the 16S rRNA of the main gut commensal species such as enterobacteria (EB), enterococci (EC), lactobacilli (LB), bifidobacteria (Bif), *Bacteroides / Prevotella* species (B/P), *Clostridium coccoides* group (Clocc), *Clostridium leptum* group (Clept), as well as the total eubacterial load (TL) were assessed (gene numbers per ng DNA). The shown data are representative for four independent experiments. Total range as well as box plots representing the 75 and 25th percentiles of the median (black bar inside the boxes) are shown.

Figure S2 | Primer sequences for molecular gut microbiota analyses.

Figure S3 | Representative photomicrographs illustrating apoptotic epithelial cell and T lymphocyte responses in the intestinal tract following PACAP treatment of mice with a human gut microbiota suffering from subacute ileitis. Subacute ileitis was induced by *T. gondii* infection of mice harboring a human gut microbiota (day 0). Starting 3 days post-infection (p.i.), mice were either treated with PACAP or placebo (PLC). Uninfected mice with a human microbiota served as control animals (Naive). Representative photomicrographs out of four independent experiments illustrate the average numbers of apoptotic epithelial cells (Casp3+; **A,C**) and of T lymphocytes [CD3+; **(B,D)**] in at least six high power fields (HPF) as quantitatively assessed in ileal **(A,B)** and colonic **(C,D)** paraffin sections applying *in situ* immunohistochemistry at day 9 p.i.

Figure S4 | Representative photomicrographs illustrating apoptotic cell and T lymphocyte responses in extra-intestinal compartments following PACAP treatment of mice with a human gut microbiota suffering from subacute ileitis. Subacute ileitis was induced by *T. gondii* infection of mice harboring a human gut microbiota (day 0). Starting 3 days post-infection (p.i.), mice were either treated with PACAP or placebo (PLC). Uninfected mice with a human microbiota served as control animals (Naive). Representative photomicrographs out of four independent experiments illustrate the average numbers of apoptotic cells [Casp3+; **(A,C)]** and of T lymphocytes [CD3+; **(B,D)]** in at least six high power fields (HPF) as quantitatively assessed in paraffin sections of *ex vivo* biopsies derived from liver **(A,B)** and lung **(C,D)** applying *in situ* immunohistochemistry at day 9 p.i.

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Prevotella histicola, A Human Gut Commensal, Is as Potent as COPAXONE® in an Animal Model of Multiple Sclerosis

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Multiple sclerosis (MS) is a demyelinating disease of the central nervous system. We and others have shown that there is enrichment or depletion of some gut bacteria in MS patients compared to healthy controls (HC), suggesting an important role of the gut bacteria in disease pathogenesis. Thus, specific gut bacteria that are lower in abundance in MS patients could be used as a potential treatment option for this disease. In particular, we and others have shown that MS patients have a lower abundance of *Prevotella* compared to HC, whereas the abundance of *Prevotella* is increased in patients that receive disease-modifying therapies such as Copaxone® (Glatiramer acetate-GA). This inverse correlation between the severity of MS disease and the abundance of *Prevotella* suggests its potential for use as a therapeutic option to treat MS. Notably we have previously identified a specific strain, *Prevotella histicola* (*P. histicola*), that suppresses disease in the animal model of MS, experimental autoimmune encephalomyelitis (EAE) compared with sham treatment. In the present study we analyzed whether the disease suppressing effects of *P. histicola* synergize with those of the disease-modifying drug Copaxone® to more effectively suppress disease compared to either treatment alone. Treatment with *P. histicola* was as effective in suppressing disease as treatment with Copaxone®, whereas the combination of *P. histicola* plus Copaxone® was not more effective than either individual treatment. *P. histicola*-treated mice had an increased frequency and number of $CD4^+FoxP3^+$ regulatory T cells in periphery as well as gut and a decreased frequency of pro-inflammatory IFN-γ and IL17-producing CD4 T cells in the CNS, suggesting *P. histicola* suppresses disease by boosting anti-inflammatory immune responses and inhibiting pro-inflammatory immune responses. In conclusion, our study indicates that the human gut commensal *P. histicola* can suppress disease as efficiently as Copaxone® and may provide an alternative treatment option for MS patients.

Keywords: multiple sclerosis, gut microbiome, Copaxone®, animal model, experimental autoimmune encephalomyelitis (EAE), HLA transgenic mice, *Prevotella histicola*, immune response

INTRODUCTION

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) and is characterized by the CNS infiltration of inflammatory cells that results in demyelination, axonal damage, and progressive neurologic disability. Collective evidence suggests that MS is caused by the destruction of the myelin sheath by aberrant T cell-mediated immune responses (1), however the etiology and pathogenesis of MS are unknown.

The interaction of both genetic and environmental factors likely plays an important role in MS pathogenesis. Genetic factors account for ∼30% of disease risk as determined from studies of identical twins (2), and among these, human leukocyte antigen (HLA) class II genes show the strongest association with disease (3). In addition, environmental factors account for 70% of disease risk (4, 5), however, how these are linked with a predisposition to, or protection from, MS is unknown. Various factors such as smoking, low vitamin D levels due to insufficient exposure to sunlight, and Epstein-Barr virus infection had been linked with a predisposition to MS. Recently, we and others have shown that dysbiosis of the gut occurs in MS patients more frequently than healthy controls (HC), indicating the gut microbiota is a potential environmental factor that contributes to the etiopathogenesis of MS (6–11). Further, MS patients have a lower abundance of the gut bacteria, *Prevotella,* compared to HC and the levels of *Prevotella* are increased in patients that receive disease-modifying therapies such as Copaxone® or Interferon beta (IFN-β) (7–9, 12). The preclinical HLA-DR3.DQ8 transgenic mouse model, which expresses the human class II genes HLA-DR3 and DQ8, develops classical experimental autoimmune encephalomyelitis (EAE) disease characterized by severe brain and spinal cord pathology and can be used to study human MS (13). To determine the significance of *Prevotella* in MS, we isolated and identified a particular strain of *Prevotella,* i.e., *Prevotella histicola (P. histicola),* and used the HLA-DR3.DQ8 transgenic mouse model of MS to demonstrate that *P. histicola* could suppress proteolipid protein $(PLP)_{91-110}$-induced EAE (14). Thus, gut bacteria can play an important role in MS pathogenesis and certain gut bacteria showing lower abundance in MS patients can be used as potential treatment option.

Copaxone® (Glatiramer acetate-GA) is an analog of myelin basic protein that is comprised of a heterogeneous mixture of polypeptides containing the four amino acids (L- glutamic acid, L-alanine, L-lysine, and L-tyrosine), and is used as a first-line disease-modifying therapy (DMTs) for the treatment of MS. Copaxone® is thought to act by suppressing antigen-specific T cell responses in the CNS (15) and by inducing the production of protective Th2 cytokines (16, 17). However, Copaxone® alone is not always efficacious in suppressing the inflammatory response in MS patients (18, 19). Therefore, there is a need to develop additional therapeutic options that can either be used alone or in combination with Copaxone® to improve treatments for MS.

Based on our data that *P. histicola* can suppress disease in a preclinical model of MS, we hypothesized that treatment with the combination of *P. histicola* and Copaxone® would have an additive effect on disease severity. In this present study, we examined the effects of the combination therapy of *P. histicola* and Copaxone® in the HLA-DR3.DQ8 transgenice mouse model of MS. In HLA-DR3.DQ8 transgenic mice, treatment with *P. histicola* suppressed EAE as efficiently as Copaxone®, whereas the combination of *P. histicola* and Copaxone® was not more effective than either treatment alone. Administration of *P. histicola,* either alone or in combination with Copaxone®, resulted in higher frequency of $CD4^+Foxp3^+$ regulatory T cells and decreased frequency of CD4 T cells that produced pro-inflammatory cytokines. Therefore, our study demonstrates that the combination of *P. histicola* and Copaxone® does not have a synergistic effect on the treatment of MS, but that *P. histicola* is as effective as Copaxone® in suppressing disease in a preclinical mouse model of MS. The disease suppression is achieved through modulation of both regulatory CD4 T cells and those producing pro-inflammatory cytokines.

MATERIALS AND METHODS

Mice

HLA-DR3.DQ8 double transgenic (DQ8 [DQA1*0103, DQB1*0302]-DR3 [DRB1*0301]) mice on the B6 background have been characterized previously (13, 20). These mice lack endogenous murine major histocompatibility complex (MHC) class II genes ($AE^{-/-}$) and express HLA-DRA1*0101, DRB1*0301, and DQA1*0103, DQB1*0302, as described previously (13, 20). These mice will be referred as HLA-DR3.DQ8 transgenic mice throughout the text. Both male and female mice (8–12 weeks of age) were utilized in this study. Mice were bred and maintained in the University of Iowa animal facility in accordance with NIH and institutional guidelines. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Iowa.

Isolation and Culture of *Prevotella histicola*

Isolation and characterization of *P. histicola* has been described previously (14). Briefly, *P. histicola* was grown at 37°C for 3 days in 5 ml of trypticase soy broth (TSB) (Hardy Diagnostics Santa Maria, USA) in an anaerobic jar with an AnaeroPack® system (Mitsubishi Gas Chemical America). The identification of *P. histicola* was confirmed by 16SrRNA-specific PCR as described previously (14).

Disease Induction And Scoring

HLA-DR3.DQ8 transgenic mice (8–12 weeks old) were immunized subcutaneously in both flanks with 25 μg of PLP_{91-110} that was emulsified in Complete Freund's Adjuvant–CFA containing *Mycobacterium tuberculosis* H37Ra (400 μg/mouse; Becton, Dickinson and Company, Sparks, MD, USA). Pertussis toxin (PTX) (Sigma Chemicals, St. Louis, MO; 100 ng) was administered i.v. at days 0 and 2 post immunization.

C57BL/6 mice were immunized subcutaneously in both flanks with MOG_{35-55} CFA/PTX as described earlier (21). Mice were observed daily for clinical symptoms and the following scoring system was used as described previously (14): 0 for normal; 1 for loss of tail tone; 2 for hind limb weakness; 3 for hind limb paralysis; 4 for hind limb paralysis and forelimb paralysis or weakness; and 5 for morbidity/death.

Treatment of HLA-DR3.DQ8 Transgenic Mice With *Prevotella histicola* and Copaxone®

We used two protocols for Copaxone® (Copaxone, GA, Teva Neuroscience) treatment: prophylactic (Copaxone® treatment given 10 days before disease induction) and therapeutic [Copaxone® treatment given during disease induction phase (7 days post EAE induction) and after onset of disease (12 days post EAE induction)].

In a prophylactic setting, HLA-DR3.DQ8 transgenic mice were divided into five groups (*P. histicola* alone, Copaxone® alone, *P. histicola* plus Copaxone®, PBS alone, and media alone). Copaxone® alone, *P. histicola* plus Copaxone® received 2 mg of Copaxone® (21) in 200 μl of incomplete Freund's adjuvant (IFA) (Becton, Dickinson and Company, Sparks, MD, USA) 10 days before EAE induction. PBS alone group received 200 μl of a PBS/IFA emulsion 10 days before EAE induction. EAE was induced by immunization with PLP_{91-110}/CFA emulsion and 7 days post EAE induction *P. histicola* alone and *P. histicola* plus Copaxone® combination group of mice were orally gavaged with live *P. histicola* (10^8 CFUs) every other day for a total of seven doses. Mice in the control group (media only) were orally gavaged with TSB media every other day for a total of seven doses.

Effect of *P. histicola* and Copaxone® were also tested in C57BL/6 mice in a prophylactic setting. Animals were divided into four groups and treated with *P. histicola* alone, Copaxone® alone, *P. histicola* plus Copaxone® or media only. *P. histicola* alone group were orally gavaged with 10^8 CFUs of live *P. histicola* every other day for a total of seven doses; Copaxone® alone group were treated subcutaneously with 100 μg of Copaxone® every other day for a total of seven doses (22); whereas combination group received both *P. histicola* and Copaxone® on alternate days as in Copaxone® or *P. histicola* only groups. Mice in the control group received 200 μl of TSB media alone by oral gavage. EAE was induced by immunization with MOG_{35-55} CFA/PTX as mentioned earlier (21).

For the therapeutic protocol, we treated mice either in disease induction phase (at disease onset) or chronic disease phase (when mice develop a score of 2). As HLA-DR3.DQ8 transgenic mice develop disease around day 7 (13), for treatment during disease induction phase, mice received 1st dose of *P. histicola* at day 7 postimmunization. In second protocol, treatment was started at day 12 post EAE induction, when mice developed a disease score of 2. Mice were divided into four groups (*P. histicola* alone, Copaxone® alone, *P. histicola* plus Copaxone®, and media alone) and treated on alternate day with *P. histicola*, Copaxone®, *P. histicola* plus Copaxone® or media as described above. All mice were evaluated for clinical EAE scores for the duration of the experiment.

Pathology

Brain and spinal cord from mice treated with *P. histicola* alone, Copaxone® alone, a combination of both *P. histicola* plus Copaxone®, or TSB media alone were histologically analyzed for inflammation and demyelination as described previously (13, 23). Briefly, mice from treated and control groups were perfused with 50 ml of Trump's fixative (0.5% glutaraldehyde + 4% paraformaldehyde) via intracardiac puncture. Brain and spinal cord were surgically removed and fixed in 10 % neutral buffered formalin (10% BFA) for 24–48 h. Spinal cord were cut into 1 mm coronal blocks, embedded in paraffin and routine processed. The resulting slide was stained with Hemotoxylin and Eosin and analyzed for pathology by a board-certified veterinary pathologist in the cortex, corpus callosum, hippocampus, brainstem, straitum, and cerebellum regions as described previously (13, 23).

Flow Cytometry

Mononuclear infiltrating cells from the brain and spinal cord were isolated using a percoll density gradient separation method as described previously (24). Mice in each treatment group were stained with antibodies to detect surface expression of CD4 (GK1.5) and CD25 (PC61) (BD Biosciences, Franklin Lakes, NJ), whereas intracellular expression of FoxP3 and IL10 were stained using an anti-Mouse/Rat FoxP3 (FJK-16s) IL10 (FES5-16E3) staining kit (eBiosciences, San Diego, CA). Intracellular staining for IL17 and IFNγ were performed using the intracellular fixation permeabilization kit and anti-mouse IL17 and IFNγ specific antibodies from eBioscience™. Cells were also stained with antibodies to detect surface expression of CD45 (30-F11) and CD4 (clone GK1.5) to gate on the leukocyte population. Gut- associated lymphoid cells were isolated and stained with antibodies as per method described previously (25).

Microbiome Analysis

Mouse fecal pellets were collected from different groups pre and postimmunization and post treatment. Microbial DNA extraction, 16S amplicon (V3-V5 region), and sequencing were done as described previously (14). R1 and R2 fastq reads were merged using Paired-End reAd merger (PEAR) (26), merged reads were converted to fasta and merged fasta sequences were process by Cloud Virtual Resource (CloVR) (27) to form operational taxonomic units (OTUs) at 97% similarity. PLS-DA score plots and histograms plots were generated using METAGENassist (28).

Statistical Analysis

Differences in the frequency of regulatory T cells or cytokine-producing CD4 T cells among mice that received treatment with *P. histicola* alone, Copaxone® alone, a combination of *P. histicola* and Copaxone®, or TSB media alone were assessed by Mann-Whitney *U*-test (**Table 1**). Average clinical EAE scores and cumulative EAE scores were compared using 2way ANOVA with multiple comparisons of the means

TABLE 1 | Effect of Copaxone® alone, *P. histicola* alone, and combination of *P. histicola* and Copaxone® on PLP_{91-110}-induced EAE in HLA-DR3.DQ8 transgenic mice.

Treatment	Disease incidence (%)	Mean onset of disease ± SD	Mean EAE Score ± SD	Number of mice with maximum severity score					
				0	1	2	3	4	5
PBS	09/09 (100%)	10.66 ± 1.30	38.57 ± 5. 37	–	–	–	–	6	3
Medium	14/14 (100%)	10.23 ± 1.37	40.07 ± 9.02	–	–	–	–	9	5
P. histicola	12/16 (75%)	12.71 ± 1.92	9.78 ± 2.09	4	7	1	4	–	–
Copaxone®	10/11 (88 %)	12.40 ± 1.39	16.22 ± 8.39	1	4	4	2	–	–
Copaxone® + *P. histicola*	10/10 (100%)	13.40 ± 1.50	17.79 ± 6.70	–	4	3	3	–	–

and non-parametric Mann-Whitney *U*-test, respectively. Statistical analyses were done with GraphPad Prism 7 (GraphPad Software, La Jolla, CA). A value of $p \leq 0.05$ was considered significant.

RESULTS

P. histicola Suppresses EAE in HLA-DR3.DQ8 Transgenic Mice as Efficiently as Copaxone®

We have shown that HLA-DR3.DQ8 transgenic mice develop severe EAE with significant brain and spinal cord pathology (13) and that EAE could be suppressed by *P. histicola* in these mice (14). In the present study, we examined whether *P. histicola* and Copaxone® can work in an additive manner to ameliorate disease in a preclinical model of MS to garner evidence for the use of this combined therapy in MS patients. We studied the effect of Copaxone® in prophylactic and therapeutic setting in combination *P. histicola* treatment in disease induction phase and after EAE induction**.**

In a prophylactic setting, mice treated with Copaxone® alone had a lower average daily clinical score compared to those treated with PBS/IFA (**Figure 1A**). Copaxone® treated mice showed a lower cumulative EAE score compared to the PBS/IFA control group. Similarly, HLA-DR3.DQ8 transgenic mice treated with *P. histicola* showed milder disease compared to those treated with media. *P. histicola*-treated mice also had a lower cumulative EAE score compared to media-treated mice (**Figure 1B** and **Table 1**). The combination of prophylactic treatment with Copaxone® and therapeutic treatment with *P. histicola* showed similar disease suppression as treatment with Copaxone® (**Figures 1A,B**, and **Table 1**). Interestingly, *P. histicola* alone group showed better disease suppression compared to combination of Copaxone® (prophylactic treatment) and *P. histicola* (therapeutic treatment).

To determine whether *P. histicola* can suppress disease in strains other than HLA transgenic mice, we investigated disease suppressive effect of *P. histicola* in C57BL/6 mice. Prophylactic treatment with *P. histicola* suppressed disease in C57BL/6 mice similar to HLA-DR3.DQ8 transgenic mice (**Figure 1C**). Additionally, Copaxone® alone and Copaxone® plus *P. histicola* treated group showed milder disease and lower average daily clinical score compared to those treated with media only (**Figure 1C**). Thus, our data indicate that treatment with *P. histicola* alone is as effective in suppressing disease as treatment with Copaxone® in HLA-DR3.DQ8 transgenic mice as well as C57BL/6 mice. Additionally, the combination of *P. histicola* plus Copaxone® is not more effective than either treatment alone.

In the prophylactic setting described above, mice received Copaxone® 2 weeks prior to start of treatment with *P. histicola*. Thus, it could be argued that Copaxone® had a dominant effect in suppressing disease because it was given prior to the start of the treatment with *P. histicola*. Therefore, we asked whether there was a synergistic effect when treatment with both Copaxone® and *P. histicola* was started at the same time. We used two therapeutic protocols with mice receiving first treatment at either day 7 postimmunization (disease induction phase) or day 12 postimmunization when mice develop a disease score of 2 (post disease development). Since in prophylactic setting media and PBS alone group showed similar effect therefore we excluded PBS alone group in therapeutic setting. Treatment with Copaxone® alone or *P. histicola* alone resulted in a lower average daily clinical score (**Figure 2A**) and the cumulative EAE score compared to treatment with media alone (**Figure 2B**). Mice that received a combination of both *P. histicola* and Copaxone® showed a slight delay in disease onset, but had a similar average daily clinical score (**Figure 2A**) and cumulative average EAE score (**Figure 2B**) compared to the groups receiving *P. histicola* or Copaxone® alone. In second protocol, HLA-DR3.DQ8 transgenic mice received first treatment when majority of mice develop score of 2 (12 days following EAE induction). Copaxone® alone, *P. histicola* alone and Copaxone® plus *P. histicola* treated group showed milder disease compared to the control media only group (**Figures 2C,D**). Thus, our data indicate that *P. histicola* is as effective at suppressing EAE when administered alone or in combination with Copaxone®, and is similar to treatment with Copaxone® alone in both preventive and therapeutic settings.

Treatment With *P. histicola* or Copaxone® Reduces Inflammation and Demyelination in The CNS

To determine whether disease suppression upon treatment with *P. histicola,* Copaxone®, or the combination of both was associated with milder CNS pathology, we analyzed CNS tissues from mice induced with EAE that received treatment with either *P. histicola* alone, Copaxone® alone, the combination

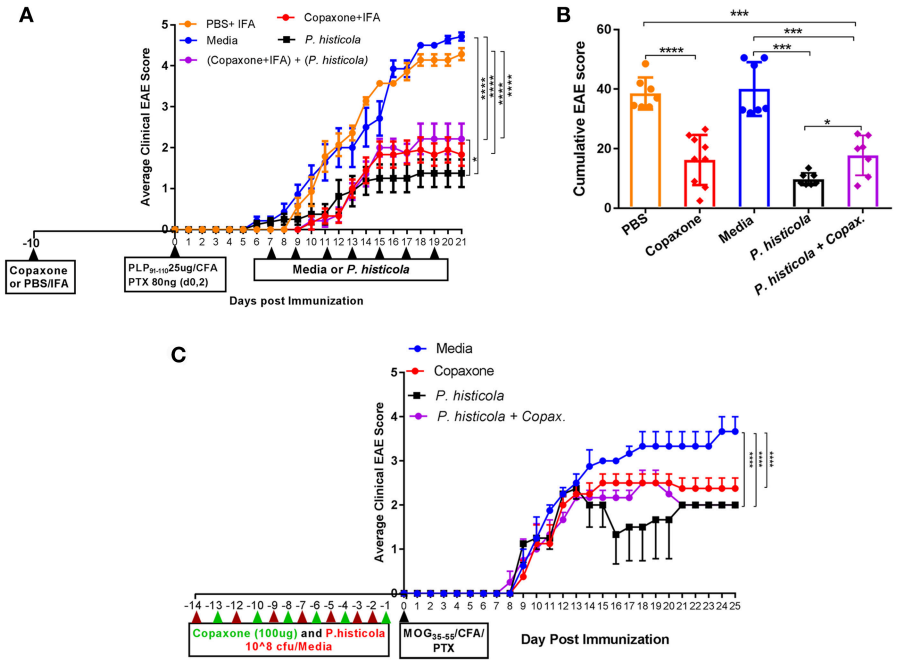

FIGURE 1 | In a prophylactic setting *P. histicola* suppressed EAE similar to Copaxone® in HLA-DR3.DQ8 transgenic and C57BL/6 mice **(A)** In a prophylactic setting, HLA-DR3.DQ8 transgenic mice were divided into five groups (*P. histicola* alone, Copaxone® alone, *P. histicola* plus Copaxone®, PBS alone, and media alone). Copaxone® alone, *P. histicola* plus Copaxone® received 2 mg of Copaxone® in 200 μl of IFA 10 days before EAE induction. PBS alone group received 200 μl of a PBS/IFA emulsion 10 days before EAE induction. EAE was induced by immunization with PLP_{91-110}/CFA (pertussis toxin (PTX) was administered on day of EAE induction and 2 days post-EAE induction) and 7 days post EAE induction *P. histicola* alone and *P. histicola* plus Copaxone® combination group of mice were orally gavaged with live *P. histicola* (10^8 CFUs) every other day for a total of seven doses. Mice in the control group (media only) were orally gavaged with TSB media every other day for a total of seven doses. Clinical scores were assessed daily for the duration of the experiment. **(B)** Cumulative EAE scores of mice treated as in **(A)**. The data presented represent 1 of 3 experiments performed at different time points ($n \geq 7$ mice per group). **(C)** C57BL/6 mice were treated with Copaxone®, *P. histicola*, or a combination of both starting day 14 prior to immunization with MOG_{35-55} CFA/PTX (with treatment administered on alternate days for a total of 14 doses, 7 doses of Copaxone® and 7 doses of *P. histicola*). Clinical scores were assessed daily for the duration of the experiment. A $^*p \leq 0.05$, $^{***}p \leq 0.0005$ and $^{****}p \leq 0.00005$ when compared to the PBS medium treated group. 2-way ANOVA Dunnett's multiple comparison test were used to calculate *p*-value among different treatment groups for average daily clinical score **(A)** and Mann-Whitney unpaired *U*-test were used to calculate *p*-value in cumulative EAE score **(B)**.

of both treatments, or media. Mice that were treated with *P. histicola* alone, or the combination of both treatments showed lower inflammation and demyelination in the brain and spinal cord compared to mice treated with media alone (**Figure 3**). Brain sections from mice treated with media showed higher number of inflammatory cells in the meningeal and stratum region (**Figure 3**). Copaxone® alone treated group showed few inflammatory regions in the brain, however the inflammation was milder than media only group. In contrast mice treated with *P. histicola* alone, Copaxone® alone, or the combination of *P. histicola* and Copaxone® showed mild meningeal inflammation (**Figure 3**). Similarly, spinal cord sections from mice that received media showed severe demyelination (i.e., loss of myelin sheath) and inflammation, whereas spinal cord sections from mice treated with *P. histicola* alone, Copaxone® alone, or the combination of *P. histicola* plus Copaxone® showed only mild inflammation in a few small areas without any significant demyelination (**Figure 3**). Thus, treatment with *P. histicola* alone, Copaxone® alone, or the combination of *P. histicola* plus Copaxone® can reduce CNS pathology in mice induced with EAE.

P. histicola Induces $CD4^+FoxP3^+$ Regulatory T cells in HLA-DR3.DQ8 Transgenic Mice

$CD4^+FoxP3^+$ regulatory T cells play an important role in suppressing EAE disease (29). Therefore, we analyzed whether $CD4^+FoxP3^+$ regulatory T cells were associated with disease suppression upon treatment with *P. histicola,* Copaxone®, or the combination of both *P. histicola* and Copaxone®. Splenocytes from mice that were induced with EAE by immunization with PLP_{91-110} and received treatment with *P. histicola* alone, Copaxone® alone, the combination of *P. histicola* plus Copaxone®, or media alone were stained for $CD4^+FoxP3^+$ regulatory T cells. Treatment with *P. histicola* alone (*P. histicola* vs. media: 11.44 ± 3.29 vs. 7.99 ± 2.19, $p > 0.01$) or the combination of *P. histicola* and Copaxone® (*P. histicola* plus Copaxone® combination vs. media: 11.25 ± 3.00 vs. 7.99 ± 2.19 vs. $p < 0.01$) resulted in a higher frequency and number of $CD4^+FoxP3^+$ regulatory T cells compared to mice treated with media alone (*P. histicola* vs. media: 1336270 ± 883218 vs. 737246 ± 265858, $P < 0.01$, *P. histicola* plus Copaxone®

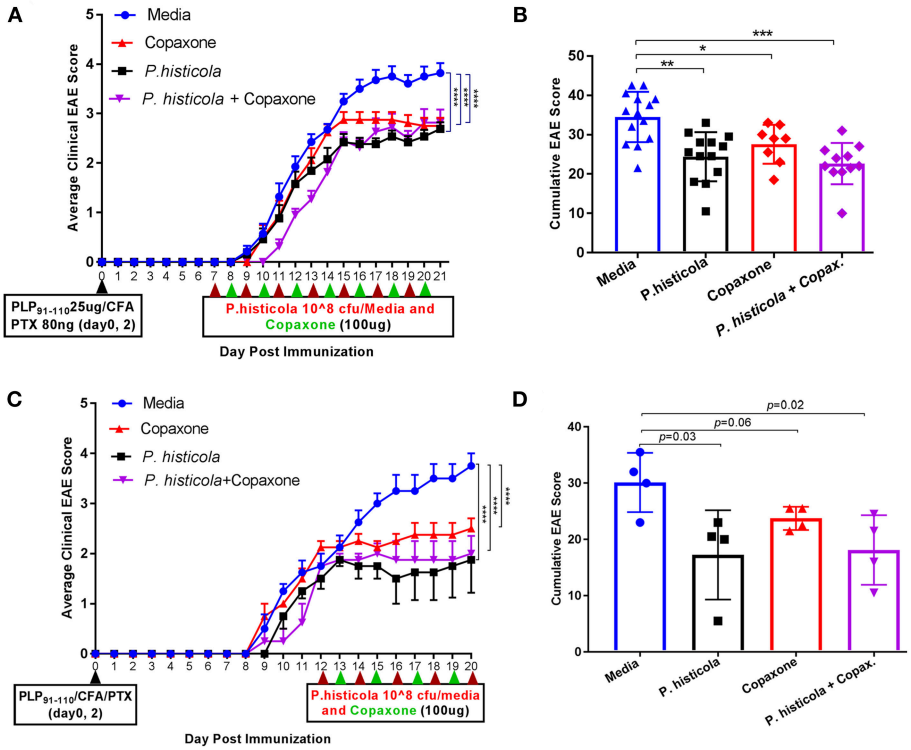

FIGURE 2 | In therapeutic setting *P. histicola* suppresses PLP_{91-110}-Induced EAE in HLA-DR3.DQ8 transgenic mice similar to therapeutic treatment of Copaxone®. **(A)** Mice were immunized with PLP_{91-110}/CFA plus pertussis toxin on days 0 and 2 of the disease induction and 1 week later mice were treated with Copaxone®, *P. histicola* or a combination of both (with treatment administered on alternate days for a total of 14 doses, 7 doses of Copaxone® and 7 doses of *P. histicola*) for 2 weeks. Clinical scores were assessed daily for the duration of the experiment. **(B)** Cumulative EAE scores of mice treated as in **(A)**. **(C)** Mice were treated with Copaxone®, *P. histicola* or a combination of both after 12 days of immunization. Clinical scores were assessed daily for the duration of the experiment. **(D)** Cumulative EAE scores of mice treated as in **(C)**. The data presented represent 1 of 3 experiments performed at different time points ($n \geq 8$ mice per group). A $^{*}p \leq 0.05$, $^{**}p \leq 0.005$, $^{***}p \leq 0.0005$ and $^{****}p \leq 0.00005$ when compared to the medium treated group. 2way ANOVA Dunnett's multiple comparisons test were used to calculate *p*-value in average clinical EAE score **(A,C)** and Mann–Whitney unpaired *U*-test were used to calculate *p*-value in cumulative EAE score **(B,D)**.

combination vs. media: 1213568 ± 425699 vs. 737246 ± 265858, $p > 0.001$) (**Figures 4A–C**). Notably, we did not observe an increased frequency of $CD4^+FoxP3^+$ regulatory T cells in mice that only received Copaxone® treatment (**Figures 4A–C**). Since, *P. histicola* is given by oral gavage, it can mediate its effect through modulation of immune cells in the gut, therefore we analyzed levels of $CD4^+FoxP3^+$ regulatory T cells and $CD4^+$ IL-10 cells in gut-associated lymphoid tissue (GALT). *P. histicola* alone treated group showed higher number of $CD4^+FoxP3^+$ regulatory T cells compared to media treated group (**Figures 4D,E**). Combination of *P. histicola plus* Copaxone® treated group only showed higher number but not frequency of $CD4^+FoxP3^+$ regulatory T cells. We could not detect any measurable level of IL-10 producing CD4+ T cells in nay groups (data not shown). Copaxone® alone group did not show any difference in $CD4^+FoxP3^+$ regulatory T cells (**Figures 4D,E**). Thus, *P. histicola* alone or in combination with Copaxone® modulates disease by inducing an anti-inflammatory immune response that is mediated by $CD4^+FoxP3^+$ regulatory T cells in gut as well as periphery. Further, our data suggests that *P. histicola* and Copaxone® might utilize different regulatory pathways to suppress disease as treatment with Copaxone® failed to increase either the frequency or number of $CD4^+FoxP3^+$ regulatory T cells in either gut or periphery (**Figure 4**).

Treatment With *P. histicola* Alone or in Combination With Copaxone® Reduces Antigen- Specific Th1 and Th17 Cytokines in the CNS of Mice Induced With EAE

CD4 T cells that produce IFNγ (Th1), IL17 (Th17), or both IFNγ and IL17 (Th1 and Th17) play an important role in EAE by inducing inflammation and demyelination in the CNS (30, 31). Therefore, we compared the frequency of IFNγ- and IL17-producing CD4 T cells among CNS-infiltrating cells in the brain and spinal cord of mice that were induced with EAE by immunization with PLP_{91-110} and treated with *P. histicola* alone, Copaxone® alone, the combination of *P. histicola* and Copaxone®, or media alone. Mononuclear cells were isolated from the brain and spinal cord of mice and stimulated with the PLP_{91-110} peptide plus Brefeldin A for 14 h (32). Mice treated with *P. histicola* had a lower frequency of both $IL17^+CD4^+$ T cells and $IFN\gamma^+CD4^+$ T cells compared to those treated with media alone ($IL17^+CD4^+$ T cells, *P. histicola* vs. media:

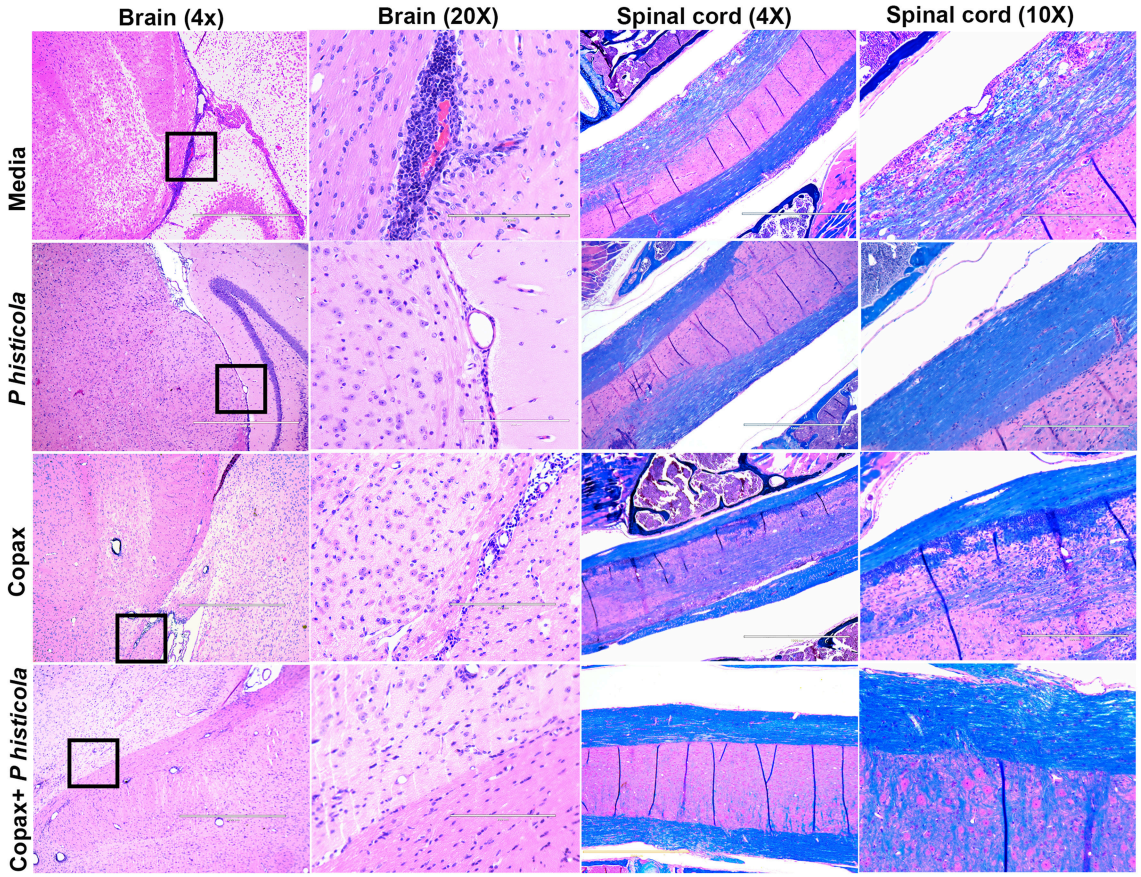

FIGURE 3 | Treatment with *P. histicola* alone, Copaxone® alone, or the combination of *P. histicola* and Copaxone® resulted in decreased inflammation and demyelination in the brain and spinal cord of HLA-DR3.DQ8 transgenic mice induced with EAE. Representative hematoxylin and eosin (H&E)-stained images of the brain or Luxol-fast blue stained images of spinal cords of mice treated with *P. histicola* alone, Copaxone® alone, *P. histicola* and Copaxone®, or media. 20X Brain section (inset black boxes in 4X) show regions of inflammation. Similarly spinal cord sections are enlarged at 10X to show regions with inflammation and demyelination. Data are representative of 3 independent experiments ($n = 5$ mice per group).

1.89 ± 0.68 vs. 4.7 ± 1.55, $p < 0.001$; IFNγ$^+$CD4$^+$ T cells, *P. histicola* vs. media: 6.1 ± 2.3 vs. 9.8 ±1.84, $p > 0.01$) (**Figures 5A,B**). In addition, mice treated with *P. histicola* had lower levels of CD4 T cells expressing both IL17 and IFNγ (**Figure 5D**). These changes were not seen with Copaxone®. Notably, mice treated with Copaxone® did not show lower levels of IL17$^+$CD4$^+$ T cells or IFNγ$^+$CD4$^+$ T cells (**Figures 5A–C**). Similar to mice treated with *P. histicola* alone, the combination of both *P. histicola* and Copaxone® decreased the frequency of IFNγ$^+$CD4$^+$ T and IL17$^+$CD4$^+$ T cells (**Figures 5A–C**). We also analyzed levels of IL-17 producing CD4$^+$ T cells in GALT to determine whether *P. histicola* or Copaxone® suppressed disease through modulation of gut resident IL17$^+$CD4$^+$ T cells. We did not observe any difference in IL-17 producing CD4$^+$ T cells among different groups (**Supplementary Figure 1**). Thus, our data suggest that treatment with *P. histicola* alone or in combination with Copaxone® decreases the frequency of IFNγ$^+$, IL17$^+$ or both IFNγ plus IL17 producing CD4 T cells in the CNS of mice with EAE.

P. histicola but Not Copaxone® Treatment Causes Restoration of Gut Microbiota

We have previously shown that *P. histicola* can mediate its protective effect through modulation of gut microbiota (14). Therefore, we first investigated whether Copaxone® treatment can also modulate gut microbiota. Fecal microbiota analysis showed that development of EAE led to a shift in microbiota profile compared to naïve mice but the group treated with *P. histicola* showed a gut microbiota profile similar to naïve mice than those with EAE (**Figure 6A**). Although treatment with Copaxone® alone cause shift in gut microbiota, it was more similar to media treated EAE group than naïve mice (**Figure 6A**). Naïve mice showed higher relative abundance of *Lactobacillus* and *P. histicola* treated group also showed higher relative abundance of *Lactobacillus* (**Figure 6B**). However, media treated EAE group showed relative loss of bacteria belonging to *Lactobacillus* genera. Interestingly Copaxone® treated group also showed relative loss of *Lactobacillus* suggesting that Copaxone® might have different mechanism than *P. histicola* in regard

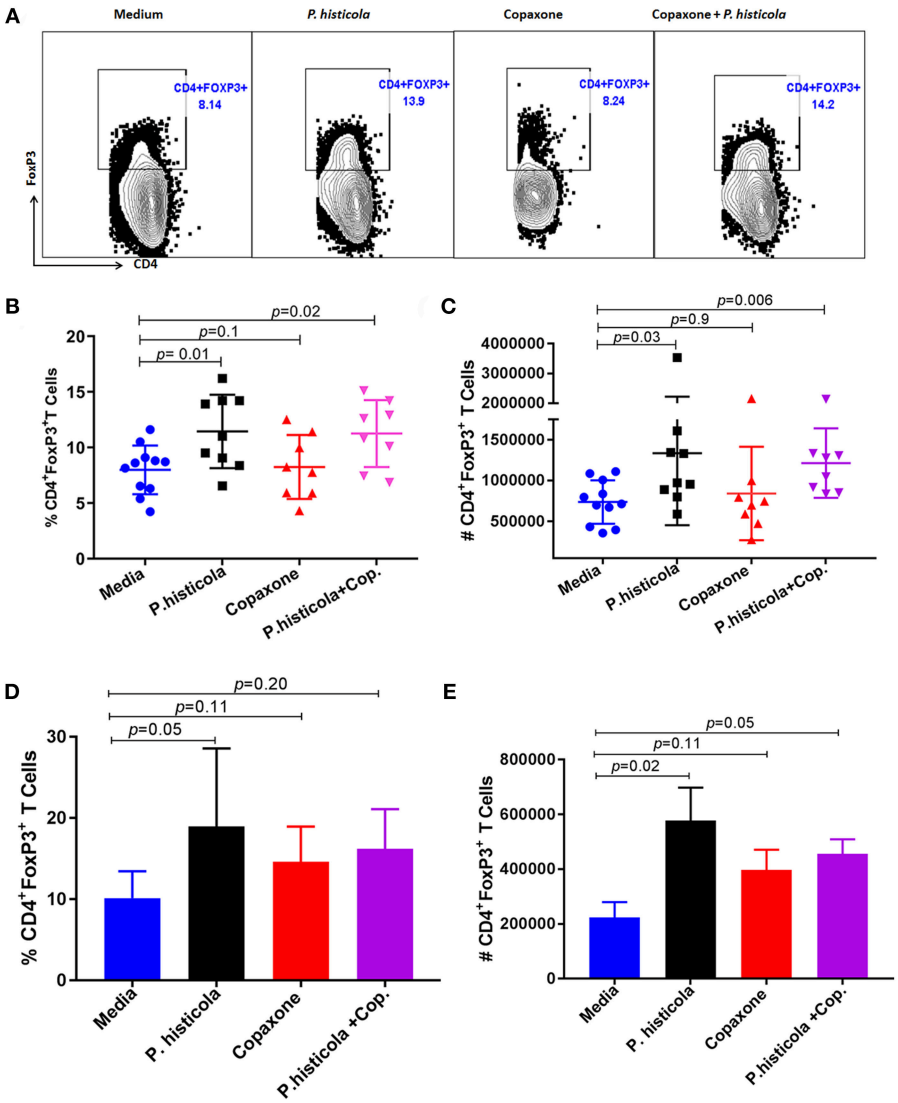

FIGURE 4 | Treatment with *P. histicola* alone or in combination with Copaxone® increases $CD4^+FoxP3^+$ regulatory T cells in the spleen and GALT. **(A)** Mice were immunized with PLP_{91-110}/CFA PTX on days 0 and 2 of the disease induction and 1 week later mice were treated with Copaxone® (7 doses), *P. histicola (7 doses),* or a combination of both (with treatment administered on alternate days for a total of 14 doses, 7 doses of Copaxone® and 7 doses of *P. histicola*). Clinical scores were assessed daily for the duration of the experiment. Representative flow cytometric plots to demonstrate $CD4^+FoxP3^+$ regulatory T cells in the spleen of mice treated with *P. histicola* alone, Copaxone® alone, *P. histicola* and Copaxone®, or media. Cells were previously gated on lymphocytes and singlets. **(B)** Frequency of $CD4^+FoxP3^+$ regulatory T cells from mice treated as in **(A)**. **(C)** Quantification of the number of $CD4^+FoxP3^+$ regulatory T cells in mice treated as in **(A)**. **(D)** Naïve mice were treated with Copaxone® (7 doses), *P. histicola* (7 doses), or a combination of both (with treatment administered on alternate days for a total of 14 doses). Gut-associated lymphoid cells were isolated from treated and control group of mice and stained with CD45, CD4 and FoxP3 antibodies. Frequency of $CD4^+FoxP3^+$ regulatory T cells from mice treated Copaxone®, *P. histicola*, *P. histicola* plus Copaxone®, and media. **(E)** Quantification of the number of $CD4^+FoxP3^+$ regulatory T cells in mice treated as in **(D)**. Error bars are presented as standard error of the mean. The *p*-value determined by Mann–Whitney unpaired *U*-test for comparing each group to media. The data presented represent 1 of 3 experiments performed at different time points ($n \geq 7$ mice per group).

to disease suppression. Finally we asked whether Copaxone® plus *P. histicola* treated group show gut microbiota profile similar to Copaxone® or *P. histicola* or different than both. As shown in **Figure 6C**, the combination of Copaxone® plus *P. histicola* treated group clustered closer to media treated EAE group than naïve mice. The combination group also showed lower levels of *Lactobacillus* compared to naïve mice (**Figure 6D**). Our gut microbiota profiling data suggests that the combination group behaved similar to Copaxone® alone group as they clustered together with media treated group characterized by relative loss of *Lactobacillus.* Thus, our data suggests that *P. histicola* might mediate its protective effect through restoration of gut microbiota to pre-immunized state whereas Copaxone® alone or combination of Copaxone® plus *P. histicola* might mediate their disease protective effect independent of gut microbiota.

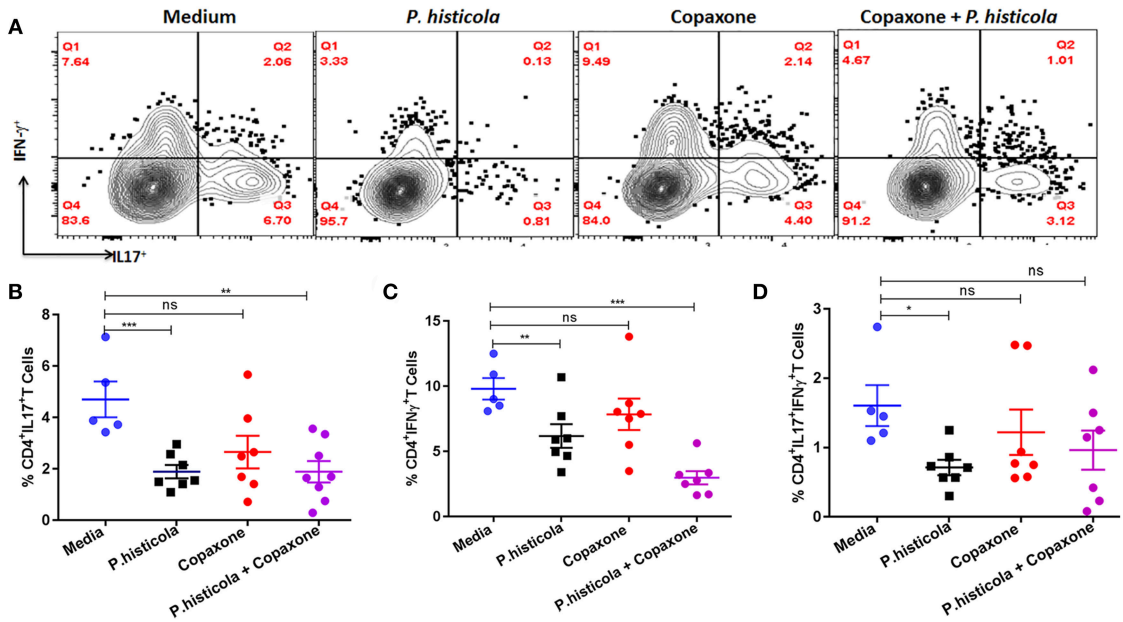

FIGURE 5 | HLA-DR3.DQ8 transgenic mice induced with EAE and treated with *P. histicola* alone or in combination with Copaxone® show a decrease in inflammatory cytokine-producing cells in the CNS. **(A)** Mice were immunized with PLP_{91-110}/CFA plus pertussis toxin on days 0 and 2 of the disease induction and 1 week later mice were treated with Copaxone® (7 doses), *P. histicola (7 doses)*, or a combination of both (with treatment administered on alternate days for a total of 14 doses, 7 doses of Copaxone® and 7 doses of *P. histicola*). Clinical scores were assessed daily for the duration of the experiment. Flow cytometric plots of $IL17^+$- or $IFN\gamma^+$-expressing mononuclear cells that were isolated from the brain and spinal cord of mice treated with *P. histicola* alone, Copaxone® alone, *P. histicola* and Copaxone®, or media. Cells were isolated and stimulated with antigen (PLP_{91-110}) plus Brefeldin A for 12 h. Plots were previously gated on $CD4^+$ cells. **(B–D)** Quantification of the frequency of $CD4^+IL17^+$T cells **(B)**, $CD4^+IFN\gamma^+$ T cells **(C)**, and $CD4^+IL17^+IFN\gamma^+$ T cells **(D)** from mice treated as in **(A)**. Cells were previously gated on lymphocytes, singlets, and $CD4^+$ cells. The data presented are the average of 2 independent experiments with $n = 4$ mice per group. The *p*-value determined by Mann–Whitney unpaired *U*-test. $^*p \leq 0.05$, $^{**}p \leq 0.005$, $^{***}p \leq 0.0005$, and "n.s." indicates not significant when compared to the media-treated group.

DISCUSSION

Here, we compared the abilities of *P. histicola* or Copaxone® to suppress EAE in the HLA-DR3.DQ8 transgenic mouse model of MS, and identify potential mechanisms by which these treatments modulate disease. Using a preclinical model of MS, we report that *P. histicola* treatment suppresses EAE as efficiently as treatment with Copaxone®, but that the combination of *P. histicola* and Copaxone® is not more effective than either treatment alone. Treatment with *P. histicola* alone or *P. histicola* plus Copaxone® increased the frequency and number of $CD4^+$ $FoxP3^+$ regulatory T cells in the spleen as well as GALT and decreased the frequency of pro-inflammatory cytokine-producing CD4 T cells in the CNS of HLA-DR3.DQ8 transgenic mice that were induced with EAE. Thus, our results provide additional evidence that certain human gut commensal bacteria play an important role in ameliorating disease.

The human microbiome project (HMP) is an NIH initiative to catalog the human microbiome (33, 34), and has thus far identified an important role for the microbiota in human health and disease (35). Consistent with this, we and others have shown that there is enrichment and/or depletion of certain gut bacteria in patients with MS compared to HC, indicating that the gut microbiota plays an important role in disease pathogenesis (7–11). This suggests that specific human gut bacteria that are depleted or found in lower abundance in MS patients may have the potential to be used in the treatment of MS. Data from our group and others showed that the *Prevotella* genus is depleted in MS patients (7–9, 12) and that treatment with the specific strain, *P. histicola,* suppresses disease in preclinical animal models of MS (14) and rheumatoid arthritis (36). Various studies of other single bacteria stains such as *Bacteroides fragilis* (37–39), *Enterococcus faecium* strain L-3 (40), *Pediococcus acidilactici* (41), and a mixture of *Lactobacillus* strains (42) have also shown efficacy in suppressing CNS-specific disease in animal models (EAE) of MS. Thus, gut commensals offer an exciting new therapeutic avenue for the MS treatment.

In MS, autoreactive CD4 T cells that are activated in the periphery, traffic to the CNS and initiate an inflammatory cascade that results in demyelination and neuronal injury. In our mouse model of MS, we observed that treatment with *P. histicola* alone, Copaxone® alone, or the combination of *P. histicola* plus Copaxone® resulted in milder pathology in the brain and spinal cord indicating that these treatments suppressed either the infiltration and/or proliferation of inflammatory cells into the CNS, thus reducing inflammation and demyelination. Although Copaxone® treated group showed few inflammatory cells in the brain tissue, it is possible that these cells are regulatory in nature. Previous studies have shown that Copaxone® can mediate its disease protective effects through induction of regulatory myeloid or $CD8^+$ T cells (21, 43). The milder pathology in the brain and spinal cord of Copaxone®-treated- mice is supported

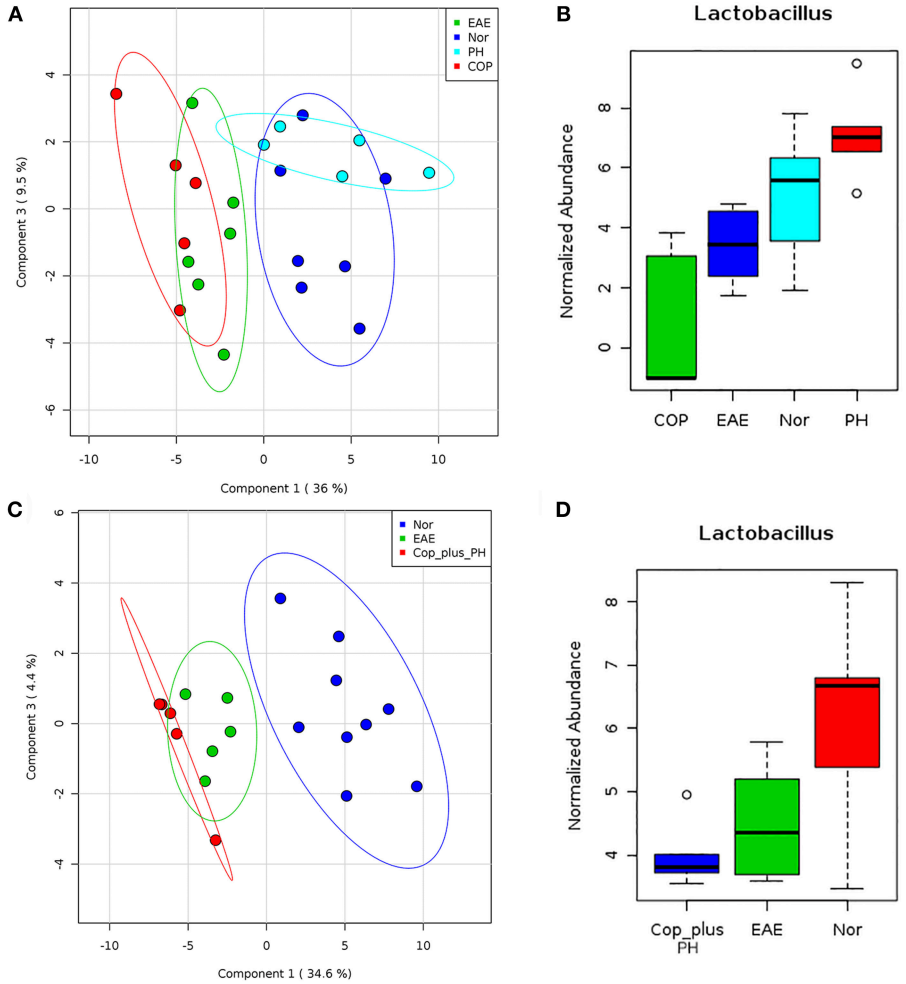

FIGURE 6 | *P. histicola* but not Copaxone® mediates disease suppression through restoration of gut microbiota **(A)** Fecal samples were collected from pre-immunized HLA-DR3.DQ8 transgenic mice (Naïve/Nor), or mice immunized for EAE (EAE) and treated with Copaxone® (Cop) or *P. histicola* (PH). Fecal DNA was extracted, 16s rRNA (V3-V5) region was amplified, and sequenced on Illumina MiSeq platform. Two dimension Partial least square- dimension analysis (PLS-DA) scores plot of fecal microbiota from different treatment groups and naïve (Nor) mice with each dot representing a mice. **(B)** Box plot showing normalized relative abundance of *Lactobacillus* among different groups. **(C)** PLS-DA scores plot showing fecal microbiota profile from pre-immunized mice (Nor) or mice immunized for EAE (EAE) or Copaxone® plus *P. histicola* (Cop_plus_PH). **(D)** Box plot showing normalized relative abundance of *Lactobacillus* among different groups. Difference among groups were analyzed using one-way ANOVA (Kruskal–Wallis rank sum test) and FDR-adjusted $p < 0.05$.

by previous study (44). Our data suggests that *P. histicola* is as effective as Copaxone® at reducing the pathology associated with disease.

MS is an inflammatory disease in which the balance between pro-inflammatory Th1/Th17 cells and anti-inflammatory $CD4^+FoxP3^+$ regulatory T cells is shifted toward a pro-inflammatory response. Therefore, potential therapeutic agents could act to suppress disease by restoring this balance toward an anti-inflammatory response, either by inducing Tregs, suppressing pro-inflammatory Th1/Th17 cells, or affecting both cell types in the periphery and in the CNS (14, 37, 38, 40–42, 45). The disease suppressive effects of *B. fragilis* and *Lactobacillus species* (mixture) in EAE were mediated by inducing $CD4^+FoxP3^+$ regulatory T cells and production of IL10 (42, 46). Similarly, we found that disease suppression mediated by *P. histicola* was associated with a higher frequency and number of $CD4^+FoxP3^+$ regulatory T cells in the gut as well as periphery. Our earlier studies also demonstrated that *P. histicola* induced $CD4^+FoxP3^+$ regulatory T cells and that these cells had higher suppressive capabilities (14). These results suggest that *P. histicola* can mediates disease suppression through induction of $CD4^+FoxP3^+$ regulatory T cells. The mechanism through which *P. histicola* induce $CD4^+FoxP3^+$ regulatory T cells is not well-understood. We hypothesize that *P. histicola* can induce Tregs through its ability to produce secondary metabolite such as short chain fatty acid (SCFA) and phytoestrogen metabolites (47). SCFA such as acetate and butyrate had been shown to induce $CD4^+FoxP3^+$ regulatory T cells (48). Certain beneficial bacteria such as *B. fragilis* can mediate their effect through

capsular polysacchride A (PSA) (39, 46, 49), however at present we do not know whether similar mechanism is true for *P. histicola*.

Collective evidence suggests that IL17- and IFNγ-producing CD4 T cells are the major pro-inflammatory cells involved in the pathogenesis of both MS and the EAE (30, 50, 51). Treatment with *P. histicola* alone or in combination with Copaxone® decreased the percentage of $IL17^+$ and $IFN\gamma^+$ $CD4^+$ T cells infiltrating the CNS of HLA-DR3.DQ8 transgenic mice. Interestingly, treatment with *P. histicola* alone also reduced infiltration of CD4 T cells that are positive for both IFNγ and IL17 ($IFN\gamma^+IL17^+$ CD4 T cells). Recent studies suggest that $IFN\gamma^+IL17^+$ CD4 T cells from humans are more pathogenic than CD4 T cells that are only producing one cytokine, i.e., either $IFN\gamma^+$ or $IL17^+$ (52). Thus, our data indicate that *P. histicola* alone or in combination with Copaxone® suppresses inflammation and demyelination by suppressing pro-inflammatory Th1 and Th17 responses. In our study, mice treated with Copaxone® did not show reduced levels of CD4 T cells expressing either IL17 or IFNγ or those expressing both $IFN\gamma^+IL17^+$ in the CNS. Although the exact mechanism by which Copaxone® suppress disease is not well-understood, previous studies have suggested it acts by diverting the immune response from a pro-inflammatory Th1 phenotype toward that of a Th2 phenotype in which cytokines such as IL5 are produced (16). Additionally, Copaxone®-induced EAE disease resistance in SJL mice is associated with the presence of Copaxone®-specific Th2 cells in the CNS and, upon adoptive transfer, these cells can be detected in the CNS of recipient mice (44). Copaxone® is considered to be an altered peptide ligand (APL) which was originally developed as an MBP mimic of myelin basic protein, a major constituent of myelin sheath. However, due to its ability to bind strongly to the MS-linked HLA-DRB1*1501 allele and induce a tolerogenic Th2 response, Copaxone® can act as a degenerate T cell antigen. Our data indicates that treatment with Copaxone® fails to alter the frequency of pathogenic Th1 and Th17 cells, whereas this population is directly affected upon administration of *P. histicola*. Failure of Copaxone® to suppress the frequency of pathogenic Th1 and Th17 cells indicate that *P. histicola* and Copaxone® might have different mechanism of action.

Ability of *P. histicola* to restore gut microbiota in EAE model, are in line with our previous finding where treatment with *P. histicola* resulted in restoration of gut microbiota to a preimmunized state (14). Interestingly, Copaxone® treated EAE group has gut microbiota similar to media treated EAE group suggesting that Copaxone® mediated disease suppression are either independent of gut microbiota or different than *P. histicola* mediated effect. Although Jangi et al. (9) have shown that MS patients on disease modifying therapies such as Copaxone® and IFNβ-1b had higher levels of *Prevotella*, it is possible that the change in *Prevotella* levels were due to IFNβ-1b and not specifically due to Copaxone®. This argument is supported by two recent studies showing that IFNβ-1b treated MS patients have higher levels of *Prevotella* (53) and MS patients receiving Copaxone® did not show any change in levels of *Prevotella* (54, 55). Thus, our gut microbiota data also points toward a non-overlapping effect of *P. histicola* and Copaxone® in regard to disease suppression in EAE model.

Our result indicates that *P. histicola* and Copaxone® have both overlapping and non-overlapping modes of action in HLA-DR3.DQ8 transgenic mice. We found that *P. histicola* alone or in combination with Copaxone® induces regulatory T cells in the spleen and suppresses inflammatory cytokine-producing CD4 T cells in the CNS of HLA-DR3.DQ8 transgenic mice. Whereas, treatment with Copaxone® suppressed disease in these mice, this was neither due to induction of $CD4^+FoxP3^+$ regulatory T cells nor due to suppression of inflammatory cytokine-producing CD4 T cells.

In summary, our findings indicate that monotherapy with *P. histicola* suppresses EAE in HLA-DR3.DQ8 transgenic mice as efficiently as Copaxone® and provides additional evidence that the gut bacteria can be used as therapeutic agents to ameliorate autoimmune inflammatory diseases such as MS. Specifically, our study suggests the possibility of using *P. histicola* in the development of a novel effective therapy for MS and other neuroinflammatory diseases.

AUTHOR CONTRIBUTIONS

AKM conceptualized the study, designed, and performed the experiments, wrote the manuscript, and gave final approval of the manuscript to be published. SS designed and performed the experiments, analyses the data, and helped with writing the manuscript. SF helped with experimental design and performing experiment. ACM performed mouse genotyping and microbiome DNA extraction. KZ and RS performed microbiome analysis. KG-C performed brain and spinal cord pathology. JM and NK helped with the study design and interpretation of the data. All authors commented on the 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/fimmu.2019.00462/full#supplementary-material

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