BEYOND PROBIOTICS: DIETARY MICROBIAL MODULATORS OF THE IMMUNE SYSTEM - EFFECTS AND MECHANISMS

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BEYOND PROBIOTICS: DIETARY MICROBIAL MODULATORS OF THE IMMUNE SYSTEM - EFFECTS AND MECHANISMS

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Table of Contents

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EDITORIAL
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Editorial: Beyond Probiotics: Dietary Microbial Modulators of the Immune System - Effects and Mechanisms

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Keywords: probiotics, prebiotics, postbiotics, SCFA, microbiota, oligosaccharides, metabolites, immune response

Editorial on the Research Topic

Beyond Probiotics: Dietary Microbial Modulators of the Immune System - Effects and Mechanisms

In recent years influential foods have emerged with force in the field of immunonutrition, with examples that include pro, pre, and postbiotics and all their combinations receiving particular attention. Probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits on the host and, among others, the immune system. After ingestion, their effects are mediated through modulation of the resident microbiota composition, bioactive molecule production, and changes in epithelial and immune intestinal cells (1). Additionally, prebiotics promote the selective growth of certain beneficial bacteria and can be used in combination with probiotic strains (synbiotics) (1). Besides the direct effect of these compounds on bacterial composition and functionality, they can also impact the immune system and host defense mechanisms through direct interaction with pathogens or with intestinal or immune cells. In recent years, a new "biotic" type has been proposed, postbiotics. This concept refers to the "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" (2). The goal of this Research Topic was to present the current state-of-the-art research on the effects of microbial modulators, also known globally as "biotics," on immune function.

This Research Topic gathers six review papers, including two minireviews, four original research papers, and one including hypothesis and theory. Among the interesting reviews included here, the paper of Liu et al. deals with probiotics, prebiotics, and postbiotics. The authors compile the effects of the three types of "biotics" on the gut microbiota and immune function, including clinical and preclinical studies demonstrating their effects and concluding that the mechanisms involved in such effects deserve further studies. Furthermore, Teame et al. introduce the concept of paraprobiotic defining the cell structural components of probiotic bacteria, to differentiate from postbiotics, i.e., metabolites of probiotics. The authors reviewed the effects of paraprobiotics and postbiotics derived from *Lactobacillus*, suggesting their potential as immunomodulators and highlighting them as a valid and safer alternative to live probiotic bacteria. In the same sense, a review concerning the role of low-doses of lactulose as a prebiotic is also included in this collection (Karakan et al.). The authors compile clinical and preclinical studies about the effects of such compounds in the composition and metabolites of the gut microbiota as well as calcium absorption. With regard to the fourth review, Rohrhofer et al. focused on the effects of dietary bioactive sphingolipids on the gut microbiota and the intestinal barrier, concluding that such compounds could combat chronic, low-grade intestinal inflammation and subsequent metabolic diseases.

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From a different point of view, the paper of De Juan et al. is a minireview that focuses on a possible mechanism of action for microbiota metabolites. Such compounds could be ligands for the aryl hydrocarbon receptor (AhR), which can act as a transcription factor that induces anti-inflammatory and immunoregulatory effects.

On the other hand, the minireview of Liu et al. summarizes the relationship between the gut and respiratory microbiota and tuberculosis and discusses the role of "biotics" in the treatment of respiratory disease. The paper evidences the importance of the gut-lung axis and that it should be considered in the treatment of respiratory diseases. In a similar point of view, the paper of Yang et al. hypothesizes the importance of a healthy microbiota in people with diabetes mellitus. The authors hypothesize about the association between low levels of microbial short-chain fatty acids (SCFA) and gut dysbiosis in diabetic people, with inflammatory responses and ulterior tumorigenesis.

The Research Topic includes four interesting research articles. One of them focuses on probiotics, in particular, the effects of *Lacticaseibacillus rhamnosus* CRL1505 in a mouse model of respiratory syncytial virus infection (Garcia-Castillo et al.). Using this model, the authors hypothesize the mechanism of action of the probiotic as involving alveolar macrophages and CD4+ cells as well interferon γ production modulation.

Two of the research articles included in this Research Topic refer to prebiotic effects. Thus, Xu et al. demonstrate that polysaccharides from a mushroom, *Tremella fuciformis*, could prevent a colonic inflammation induced in mice. The authors consider the mechanisms involved in such protective effects to be driven by changes in the intestinal microbiota. Another preclinical study demonstrated the protective effect of a prebiotic from citrus pulp against the weaning stress of piglets (Uerling et al.). The authors showed the effects of this prebiotic in the intestinal health of piglets as well as the influence on their microbiota.

Finally, a promising clinical study was carried out in Uganda by comparing the effects of the administration of probiotic yogurt or that of milk on the incidence rate for common cold symptoms and the skin infection symptoms of children aged 3 to 6 years old (Westerik et al.). Although the authors recognize that the study had some limitations, they observed a positive trend of yogurt against the incidence of common cold and skin infections, therefore opening up new perspectives for further studies.

This Research Topic includes interesting papers focusing on "biotics" from different types and origins, demonstrating that a large number of products can be included in this category. In addition, overall, they highlight the role of "biotics" not only in intestinal health but also in respiratory and cutaneous infections. Further studies are encouraged to expand the knowledge of these bioactive compounds, to establish their modes of action, and above all to demonstrate, by means of clinical studies, the importance of such products in health.

AUTHOR CONTRIBUTIONS

All authors contributed equally to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Alveolar Macrophages Are Key Players in the Modulation of the Respiratory Antiviral Immunity Induced by Orally Administered *Lacticaseibacillus rhamnosus* CRL1505

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The oral administration of *Lacticaseibacillus rhamnosus* CRL1505 differentially modulates the respiratory innate antiviral immune response triggered by Toll-like receptor 3 (TLR3) activation in infant mice, improving the resistance to Respiratory Syncytial Virus (RSV) infection. In this work, by using macrophages depletion experiments and a detailed study of their production of cytokines and antiviral factors we clearly demonstrated the key role of this immune cell population in the improvement of both viral elimination and the protection against lung tissue damage induced by the CRL1505 strain. Orally administered *L. rhamnosus* CRL1505 activated alveolar macrophages and enhanced their ability to produce type I interferons (IFNs) and IFN-γ in response to RSV infection. Moreover, an increased expression of *IFNAR1, Mx2, OAS1, OAS2, RNAseL,* and *IFITM3* was observed in alveolar macrophages after the oral treatment with *L. rhamnosus* CRL1505, which was consistent with the enhanced RSV clearance. The depletion of alveolar macrophages by the time of *L. rhamnosus* CRL1505 administration abolished the ability of infant mice to produce increased levels of IL-10 in response to RSV infection. However, no improvement in IL-10 production was observed when primary cultures of alveolar macrophages obtained from CRL1505-treated mice were analyzed. Of note, alveolar macrophages from the CRL1505 group had an increased production of IL-6 and IL-27 suggesting that these cells may play an important role in limiting inflammation and protecting lung function during RSV infection, by increasing the maturation and activation

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of Treg cells and their subsequent production of IL-10. In addition, we provided evidence of the important role of $CD4^+$ cells and IFN-γ in the activation of alveolar macrophages highlighting a putative pathway through which the intestinal and respiratory mucosa are communicated under the influence of *L. rhamnosus* CRL1505.

Keywords: *Lacticaseibacillus rhamnosus* CRL1505, immunobiotics, TLR3, viral immunity, Respiratory Syncytial Virus, alveolar macrophages

INTRODUCTION

The effect of the intestinal microbiota on the immune responses in the respiratory tract and its impact on the outcome of viral infections has been explored during the last decade (1–3). Those studies demonstrated that the signals provided by the intestinal microbiota act at multiple levels in the respiratory mucosa stimulating an antiviral state in non-immune cells and innate immune cells that would allow an efficient control of viral replication early during the infection. Although these effects of the intestinal microbiota have been documented mainly for Influenza Virus (IFV) infection (1–3), it should be considered that the cellular and molecular mechanisms involved in the innate antiviral immune response are not virus specific and therefore, they are similar for all the respiratory viruses. Then, the beneficial microbes in the intestinal tract may favorably influence the innate immune responses to other respiratory viruses as well. In this regard, it was recently demonstrated that a high-fiber diet improved the production of acetate by the intestinal microbiota of mice and that this metabolic product modulated the activity of respiratory interferon (IFN)-β and increased the expression of interferon-stimulated genes (ISGs) in the lung (4). The immunological changes induced in the respiratory tract by the dietary treatment significantly increased the resistance of mice to the challenge with Respiratory Syncytial Virus (RSV).

The studies with germ-free as well as antibiotic-treated mice recolonized with bacteria demonstrated that the changes induced by the intestinal microbiota in the respiratory antiviral immune response are reversible and more importantly, tunable (1–3, 5). Moreover, it was shown that not all commensal bacteria could contribute equally to the antiviral immunocompetence in the lung (1). These findings have opened the possibility of exploring particular strains of beneficial bacteria with immunomodulatory capacities, referred to as immunobiotics, in order to increase antiviral defenses in the respiratory tract. Then, several immunomodulatory beneficial microbial strains mainly from the Lactobacillus and Bifidobacterium species; have been tested in their capacities to modulate the respiratory antiviral immune response when orally administered (6, 7). A growing number of studies have examined the effect of immunobiotic nutritional interventions on the incidence, the duration and severity of respiratory infections in humans. Several clinical trials, systematic reviews and meta-analyses have suggested that immunobiotics may be effective in improving the resistance of children against viral respiratory infections such as the common cold and influenza-like symptoms (8, 9). Interestingly, immunobiotic intervention was shown to reduce the episodes of viral respiratory infections even in asthmatic children (10). Collectively, research has shown that some immunobiotic strains are capable of making a difference in the host's response to respiratory viral infections. However, there is limited information regarding the cellular and molecular mechanisms involved in the beneficial effects of each particular immunobiotic strain. Those kind of studies are necessary to provide solid scientific basis to promote the use of immunobiotics in the prevention of respiratory viral infections.

In a randomized controlled trial in children, it was demonstrated that the immunobiotic strain *Lacticaseibacillus rhamnosus* CRL1505 (Basonym, *Lactobacillus rhamnosus* CRL1505) (11), administered in a yogurt, improved mucosal immunity and reduced the incidence and severity of viral intestinal and respiratory infections (12). Since this finding in children, our laboratory had perform studies by using *in vivo* and *in vitro* models to characterize the antiviral properties of *L. rhamnosus* CRL1505. We demonstrated that the oral administration of the CRL1505 strain was capable of improving the resistance of infant mice to RSV infection (13, 14). We found that *L. rhamnosus* CRL1505 differentially regulated the respiratory innate antiviral immune response triggered by the activation of Toll-like receptor 3 (TLR3), improving the resistance to RSV infection. The study of the immunological mechanisms involved in the protective effect induced by *L. rhamnosus* CRL1505 revealed a key role for respiratory IFN-β, IFN-γ, and interleukin (IL)-10. Our results showed that the increase of the three cytokines in the respiratory tract induced by the oral CRL1505 treatment was involved in the reduction of lung RSV titers and inflammatory-mediated lung tissue injury. Moreover, our studies provided us with preliminary information that indicate that $CD11c^+SiglecF^+$ alveolar macrophages would actively participate in the beneficial effects induced by *L. rhamnosus* CRL1505 (13, 14). However, the exact role of resident alveolar macrophages in the immunomodulatory effects of the CRL1505 strain have not been investigated in detail.

Abbreviations: BAL, Broncho-alveolar lavages; CXCL1, Chemokine (C-X-C motif) ligand 1; CLP, Clodronate-containing liposomes; DMEM, Dulbecco's modified Eagle's medium; ELISA, Enzyme-linked immunosorbent assay; ELP, Empty liposomes; HRP, Horseradish peroxidase; IFV, Influenza Virus; IL, Interleukin; IFN, Interferon; IFNAR1, IFN-alpha/beta receptor alpha chain; Mx, IFN-induced GTP-binding protein Mx; ISGs, Interferon-stimulated genes; IFITM3, IFN-induced transmembrane protein 3; LDH, Lactate dehydrogenase; MRS, Man-Rogosa-Sharpe; MAMPs, Microbial-associated molecular patterns; MOI, Multiplicity of infection; OAS, 2'-5'-oligoadenylate synthetase; PRRs, Pattern recognition receptors; PBS, Phosphate buffer saline; RSV, Respiratory syncytial virus; RNAseL, Ribonuclease L; TLR3, Toll-like receptor 3.

Considering this background, in this work we aimed to further advance in the characterization of the beneficial effects of *L. rhamnosus* CRL1505 in the context of respiratory RSV infection by evaluating whether their immunomodulatory properties are dependent on alveolar macrophages function. The role of alveolar macrophages in the differential cytokine profile induced in the respiratory tract by orally administered *L. rhamnosus* CRL1505, as well as in its ability to increase the resistance to RSV infection was evaluated.

MATERIALS AND METHODS

Microorganisms

Lacticaseibacillus rhamnosus CRL1505 and *Lactiplantibacillus plantarum* CRL1506 (Basonym, *Lactobacillus plantarum* CRL1505) (11) were obtained from the CERELA culture collection (Chacabuco 145, San Miguel de Tucumán, Argentina). Both lactobacilli cultures were kept freeze-dried. Lactobacilli were cultured for 12 h at 37°C (final log phase) in Man-Rogosa-Sharpe broth (MRS, Oxoid). The bacteria were harvested by centrifugation at 3,000 g for 10 min, washed three times with sterile 0.01 mol/L phosphate buffer saline (PBS, pH 7.2), and resuspended in sterile 10% non-fat milk.

Animals and Treatments

Infant (3-week-old) BALB/c mice were obtained from the closed colony kept at CERELA (San Miguel de Tucumán, Argentina). Animals were housed in plastic cages at room temperature and the assays for each parameter studied were performed in 5–6 mice per group for each time point. Three groups of mice were used: CRL1505- and CRL1506-treated mice and PBS-treated controls. *L. rhamnosus* CRL1505 and *L. plantarum* CRL1506 strains were orally administered to infant mice for five consecutive days. Mice were deprived of water for 4 h and the immunobiotic strains were given at a dose of 10^8 cells/mouse/day in a minimum volume of drinking water containing 10% of non-fat milk to animals in individual cages (13, 14). The treated groups and the PBS-treated control group were fed a conventional balanced diet *ad libitum*.

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Guidelines for Animal Experimentation of CERELA. The CERELA Institutional Animal Care and Use Committee prospectively approved this research under the protocol BIOT-CRL-18. All efforts were made to minimize the number of animals and their suffering. No signs of discomfort or pain were observed before mice reached the endpoints. No deaths were observed before mice reached the endpoints.

Blood and respiratory tissue samples were obtained from mice after the intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) according to the recommendations of the CERELA Institutional Animal Care and Use Committee.

Poly(I:C) Administration and RSV Infection

Administration of the TLR3 agonist poly(I:C) (Sigma-Aldrich) was performed 2 days after the last day of lactobacilli treatments. Mice received 100 μl of PBS containing 250 μg poly(I:C) (equivalent to 10 mg/kg body weight), that was administered dropwise, via the nares (13, 15, 16). Control animals received 100 μl of PBS. Mice received three doses of poly (I:C) or PBS with 24 h rest period between each administration.

Human RSV strain A2 was grown in Vero cells as described previously (13–15). Briefly, Vero cells were grown in Dulbecco's modified Eagle's medium (DMEM) and infected with RSV at a multiplicity of infection (MOI) of 1 in 5 ml for 3 h at 37°C, 5% CO_2. After infection, 7 ml of DMEM with 10% fetal bovine serum (Sigma, Tokyo, Japan), 0.1% penicillin-streptomycin (Pen/Strep) (Sigma, Tokyo, Japan), and 0.001% ciprofloxacin (Bayer) was added to the flask, and cells were incubated until extensive syncytium formation was detected. Then, Vero cells were scraped, sonicated and cell debris was removed by centrifugation at 700 g for 10 min at 4°C. Virus supernatant was sucrose density gradient purified and stored in 30% sucrose at −80°C. For *in vivo* infection, mice were challenged with 10^6 PFU (Plaque forming units) of RSV by the nasal route (13–15). Viral challenge was performed 2 days after the last day of lactobacilli treatments. Lung RSV titers and tissue damage were evaluated 2 days after viral infection. The RSV immunoplaque assay was performed as described previously (14–16), briefly lungs were homogenized and diluted tissue clarified supernatants were added to Vero cells monolayers. Samples were run in triplicate. After 3 h of incubation (37°C, 5% CO_2) supernatants were removed and fresh medium (DMEM, 10% FBS, 0.1% Pen- Strep, 0.001% ciprofloxacin) was added. Monolayers were fixed with ice cold acetone: methanol (60:40) when extensive syncytia were observed. Primary RSV anti-F (clones 131-2A; Chemicon), anti-G (Mouse monoclonal [8C5 (9B6)] to RSV glycoprotein, Abcam) and secondary horseradish peroxidase anti-mouse immunoglobulin (Anti-mouse IgG, HRP-linked Antibody #7076, Cell signaling Technology) antibodies were used. Individual plaques were developed using a DAB substrate kit (ab64238, Abcam) following manufacture's specifications. Results were expressed as $\log_{10}$ PFU/g of lung.

Lung Injury Parameters

Broncho-alveolar lavages (BAL) samples were obtained as described previously (16, 17). Briefly, the trachea was exposed and intubated with a catheter, and 2 sequential lavages were performed in each mouse by injecting sterile PBS. The recovered fluid was centrifuged for 10 min at 900 g; and frozen at −70°C for subsequent analyses.

Albumin content, a measure to quantitate increased permeability of the bronchoalveolar–capillarity barrier, and lactate dehydrogenase (LDH) activity, an indicator of general cytotoxicity, were determined in the acellular BAL fluid. Albumin content was determined colorimetrically based on albumin binding to bromcresol green using an albumin diagnostic kit (Wiener Lab, Buenos Aires, Argentina). LDH activity, expressed as units per liter of BAL fluid, was determined by measuring the formation of the reduced form of nicotinamide adenine dinucleotide (NAD) using the Wiener reagents and procedures (Wiener Lab).

In vivo Depletion of Alveolar Macrophages

For depletion of alveolar macrophages, mice were inoculated intranasally with 50 µl of clodronate (dichloromethylene-bisphosphonate)-containing liposomes (CLP; Clophosome, Stratech, United Kingdom) as described elsewhere (18). Mice were treated with CLP for two consecutive days. Optimal conditions of alveolar macrophages depletion were determined by differential counting of BAL cells (**Figure 1**). An equal treatment with empty liposomes (ELP) served as controls.

The total number of leukocytes and differential cell counts in BAL samples were performed as described previously (13, 15, 16). Briefly, the total number of leukocytes was determined with a hemocytometer. Differential cell counts were performed by counting 200 cells in blood smears stained with May Grunwald-Giemsa.

Alveolar Macrophages Primary Cultures

Primary cultures of murine alveolar macrophages was performed as described elsewhere (19, 20). Macrophages were obtained from infant mice via bronchoalveolar lavages by using 1 ml of warm sterile PBS containing 5 mM EDTA. Macrophages were transferred to new sterile tubes, washed twice in sterile PBS, and resuspended in RPMI 1640 medium with 10% FBS, 1 mM L-glutamine, and 100 U/ml penicillin-streptomycin. BAL cells were seeded in 24-well-plates at a density of 10^5 cells/well and incubated for 2 h at 37°C in 5% CO_2 to promote adherence. Non-adherent cells were washed and macrophages were maintained in culture in RPMI 1640 medium with 10% FBS, 1 mM L-glutamine, and 100 U/ml penicillin-streptomycin at 37°C in 5% CO_2 for 24 h before stimulation. Alveolar macrophages were stimulated with poly(I:C) (50 ug/ml), or RSV (MOI of 5). Supernatants were collected before (basal conditions) and 24 h after stimulations, for cytokines analysis. In addition, the mRNA was extracted from alveolar macrophages 12 h after RSV challenge for the evaluation of cytokines and antiviral factors gene expressions.

Cytokine Concentrations in BAL and Culture Supernatants

BAL samples were obtained as described previously (16, 17). The samples were frozen at −70°C for subsequent cytokine analyses. IFN-β (Mouse IFN-beta ELISA Kit, sensitivity: 15.5 pg/ml), IFN-γ (Mouse IFN-gamma Quantikine ELISA Kit, sensitivity: 2 pg/ml), IL-6 (Mouse IL-6 Quantikine ELISA Kit, sensitivity: 1.8 pg/ml), IL-10 (Mouse IL-10 Quantikine ELISA Kit, sensitivity: 5.2 pg/ml), IL-12 (Mouse IL-12 p70 DuoSet ELISA, sensitivity: 1.5 pg/ml) and IL-27 (Mouse IL-27 p28/IL-30 Quantikine ELISA Kit, sensitivity: 4.7 pg/ml), IL-17 (Mouse IL-17 Quantikine ELISA Kit, sensitivity 5 pg/ml), and the IL-8 mouse homolog chemokine KC or chemokine (C-X-C motif) ligand 1 (Mouse CXCL1/KC DuoSet ELISA, sensitivity 2.3 pg/ml) concentrations in BAL and culture supernatants samples were measured with commercially available enzyme-linked immunosorbent assay (ELISA) technique kits following the manufacturer's recommendations (R&D Systems, MN, USA).

Quantitative Expression Analysis by Real-Time PCR

Alveolar macrophages were obtained as described above and total RNA was isolated from each sample using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. After the chloroform step, the aqueous phase–containing RNA was further processed using the RNeasy Micro kit (QIAGEN) according to the manufacturer's instructions.

Two-step real-time quantitative PCR was performed to characterize the expression of *IFN-α, IFN-β, IFN-γ,* IFN-alpha/beta receptor alpha chain (*IFNAR1*), *IFN-λ1, IFN-λ2/3,* the IFN-induced GTP-binding protein Mx1 (*Mx1*), *Mx2,* the 2′-5′-oligoadenylate synthetase 1 (*OAS1*), *OAS2,* the ribonuclease L (*RNAseL*), and the IFN-induced transmembrane protein 3 (*IFITM3*) genes in cultured alveolar macrophages. All cDNAs were synthesized using a Quantitect reverse transcription (RT) kit (Qiagen, Tokyo, Japan) according to the manufacturer's recommendations. Real-time quantitative PCR was carried out using a 7300 real-time PCR system (Applied Biosystems, Warrington, United Kingdom) and the Platinum SYBR green qPCR SuperMix uracil-DNA glycosylase (UDG) with 6-carboxyl-X-rhodamine (ROX) (Invitrogen). The primers used in this work are given in the (**Supplementary Table 1**). The PCR cycling conditions were 2 min at 50°C, followed by 2 min at 95°C, and then 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. The reaction mixtures contained 5 µl of sample cDNA and 15 µl of master mix, which included the sense and antisense primers. Expression of β-actin was used to normalize cDNA levels for differences in total cDNA levels in the samples.

Flow Cytometry Analysis

Single cells from BAL samples were prepared as previously described (14, 16, 17). Erythrocytes were depleted by hypotonic lysis and the cells were washed with RPMI medium supplemented with 10% heat-inactivated fetal calf serum (FCS). Cells were counted using Trypan Blue exclusion and then resuspended at an appropriate concentration of 5×10^6 cells/ml.

BAL cell suspensions were pre-incubated with anti-mouse CD32/CD16 monoclonal antibody (Fc block) for 15 min at 4°C. Cells were incubated in the antibody mixes for 30 min at 4°C and washed with FACS buffer. Then, cells were stained with fluorochrome-conjugated antibodies against CD11c (APC), SiglecF (PE) (BD Bioscience), CD45 (FITC) (eBioscience), and MHC-II (PerCP) (Thermo Fisher Scientific). Cells were then acquired on a BD FACSCalibur™ flow cytometer (BD Biosciences) and data were analyzed with FlowJo software (TreeStar). The total number of cells in each population was determined by multiplying the percentages of subsets within a series of marker negative or positive gates by the total cell number determined for each BAL sample (14, 16, 17).

Blocking Experiments

In order to evaluate the role of IFN-γ in the immunomodulatory capacity *L. rhamnosus* CRL1505, anti-IFN-γ blocking antibodies were used. Different groups of mice were orally treated with *L. rhamnosus* CRL1505 for 5 consecutive days at a dose of 10^8 cells/mouse/day as described above. On days 1, 3, and

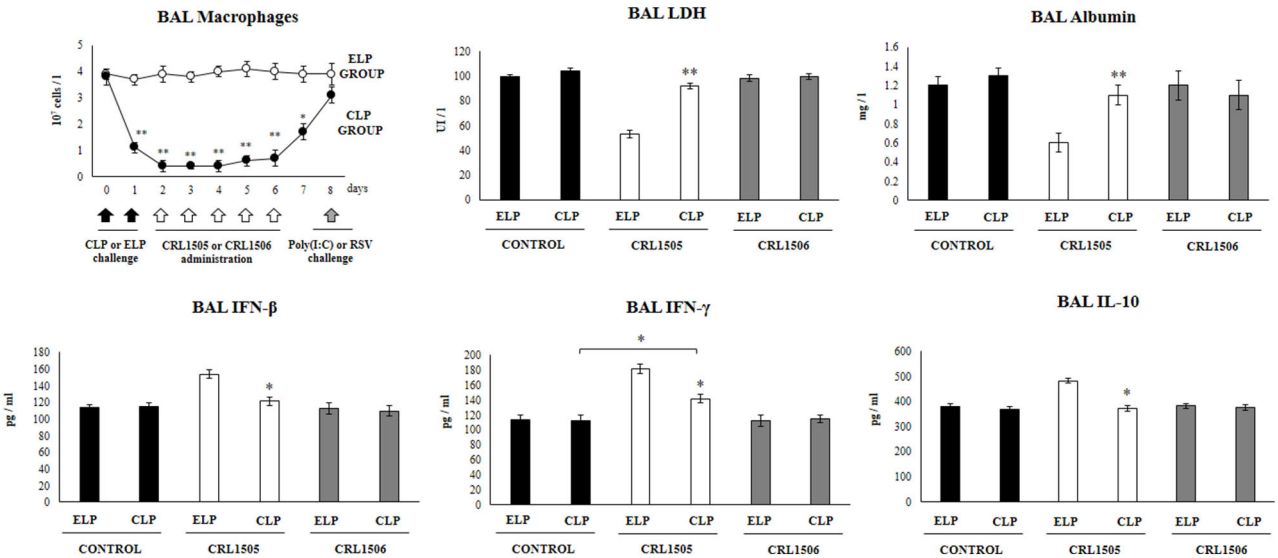

FIGURE 1 | Effect of alveolar macrophages depletion on the ability of *Lacticaseibacillus rhamnosus* CRL1505 to modulate the respiratory immune response triggered by poly(I:C) treatment. Infant mice were nasally treated with clodronate-containing liposomes (CLP) during 2 days (days 0 and 1) to induce the depletion of alveolar macrophages. One day after the last CPL administration mice were orally treated with *L. rhamnosus* CRL1505 or *Lactiplantibacillus plantarum* CRL1506 (10^8 cell/mouse/day) during five consecutive days (days 2–6) and challenged with three once-daily doses of poly(I:C) (days 8, 9, and 10). Mice treated with empty liposomes (ELP), CRL1505 or CRL1506 and then challenged with poly(I:C) were used as controls. Two days after the last poly(I:C) administration lactate dehydrogenase (LDH) activity, albumin concentrations, and the levels of interferon (IFN)-β, IFN-γ, and interleukin (IL)-10 in broncho-alveolar lavages (BAL) were evaluated. The results represent data from three independent experiments. Asterisks indicate significant differences between the respective ELP and CLP groups. Asterisks in black lines indicate significant differences between the indicated groups. *$P < 0.05$, **$P < 0.01$.

5 mice were injected intraperitoneally with 20 ug of purified anti-IFN-γ antibodies (LEAF™ Purified anti-mouse IFN-γ IgG1 antibody, #505706 BioLegend) or 80 ug of isotype control antibodies (LEAF™ Purified Rat IgG1, % Isotype Ctrl, LEAF™ Purified Rat IgG1, % Isotype Ctrl, BioLegend) as we described previously (13). For CD4 depletion, mice were intraperitoneally injected with 200 μg of anti-α-CD4 antibodies (Rat IgG2b, clone GK1.5; #AB1107636, BioXcell) or IgG isotype control antibodies (rat IgG2b isotype, clone LTF-2, #BE0090, BioXcell) on days 1, 3, and 5.

Statistical Analysis

Experiments were performed in triplicate and results were expressed as mean ± standard deviation (SD). After verification of the normal distribution of data, 2-way ANOVA was used. Tukey's test (for pairwise comparisons of the means) was used to test for differences between the groups. Differences were considered significant at $p < 0.05$.

RESULTS

Effect of Alveolar Macrophages Depletion on the Capacity of *L. rhamnosus* CRL1505 to Modulate the Respiratory Immune Response Triggered by Poly(I:C)

The nasal administration of liposomes containing toxic substances such as clodronate is a widely used technic to evaluate the role of alveolar macrophages in respiratory immune responses (18, 20). Here, we used this experimental approach to evaluate the role of alveolar macrophages in the immunomodulatory effects of orally administered *L. rhamnosus* CRL1505. In addition, we used the immunobiotic strain *L. plantarum* CRL1506 for comparison. Our previous studies demonstrated that the oral administration of the CRL1505 or CRL1506 strains to mice are able to beneficially modulate intestinal antiviral immunity (21, 22). However, only *L. rhamnosus* CRL1505 exerts a beneficial effect at a distance by modulating respiratory immunity.

In our hands, the nasal administration of clodronate-containing liposomes (CLP) significantly reduced the number of macrophages in BAL samples for a period of 6 days. The number of BAL macrophages started to recover from day 7 (**Figure 1**). This effect was not observed in infant mice nasally treated with empty liposomes (ELP) in which BAL macrophages numbers were normal during all the assessed period. These results indicated that CLP treatment is useful for evaluating the role of alveolar macrophages in the immunomodulatory effect of the CRL1505 strain in our experimental models, since these immune cells are decreased at the time of lactobacilli administration, while their number return to normality when the poly(I:C) or RSV challenges occur.

We and others demonstrated that the nasal administration of the TLR3 agonist poly(IC) to mice induce an inflammatory

response and lung functional changes that are similar to those caused by RSV (13, 14). Therefore, we used the nasal administration of the dsRNA analog poly(I:C) to mimic the pro-inflammatory and physiopathological consecuences of RNA viral infections in the lung. In addition, we used the BAL biochemical markers albumin and LDH to assess the lung damage. Albumin is not detected normally in BAL samples and the increase of this protein in the respiratory tract is an indicator of an increased permeability of the bronchoalveolar–capillarity barrier. On the other hand, the increase of the intracellular enzyme LDH in BAL samples is an indicator of cytotoxicity (13, 14).

It was observed that in infant mice treated with ELP and challenged with poly(I:C) the levels of BAL LDH and albumin increased significantly after TLR3 activation (**Figure 1**). It was also observed that in animals treated with ELP and *L. rhamnosus* CRL1505 and subsequently challenged with poly(I:C) the levels of BAL LDH and albumin were significantly lower than those found in their respective control group (ELP control mice) (**Figure 1**). These results clearly indicate that treatment with ELP does not modify the number or functionality of alveolar macrophages, since both the effect of poly(I:C) and the CRL1505 strain in ELP-treated mice are similar to those previously described in conventional mice (13, 14).

The administration poly(I:C) to CLP-treated control mice significantly increased the values of lung damage markers with no differences respect to ELP control animals. It was also observed that in infant mice treated with CLP, the administration of *L. rhamnosus* CRL1505 was not able to reduce the levels of BAL LDH and albumin after the challenge with poly(I:C) (**Figure 1**). Mice treated with ELP or CLP and *L. plantarum* CRL1506 showed lung injury markers values that were not different from their respective controls (**Figure 1**).

The levels of IFN-β, IFN-γ, and IL-10 in BAL were also determined after administration of poly(I:C), in infant mice treated with CLP or ELP (**Figure 1**). The administration of the TLR3 agonist induced significant increases in the levels of BAL IFN-β, IFN-γ, and IL-10 in the ELP control mice, which were similar to those previously reported (13, 14). It was also observed that *L. rhamnosus* CRL1505 induced significant increases in the levels of BAL IFN-β and IFN-γ as well as in IL-10 after the administration of poly(I:C) in ELP-treated mice compared with ELP controls (**Figure 1**).

In control infant mice treated with CLP, the values of the three cytokines were similar to the found in the ELP control group after the stimulation with poly(I:C). The treatment of CLP mice with *L. rhamnosus* CRL1505 increased the levels of IFN-γ, but the values of this cytokine did not reach the levels observed in mice treated with ELP and the CRL505 strain (**Figure 1**). It was also observed that in infant mice treated with CLP, the administration of *L. rhamnosus* CRL1505 was not able to increase the levels of BAL IFN-β or IL-10 after the challenge with poly(I:C) when compared to

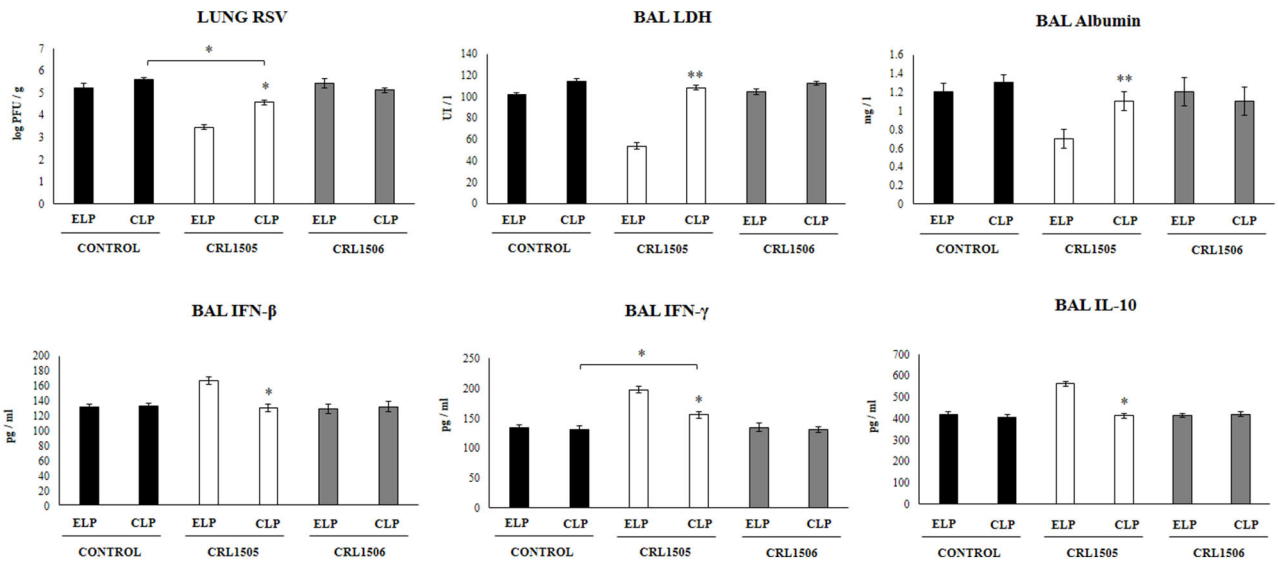

FIGURE 2 | Effect of alveolar macrophages depletion on the ability of *Lacticaseibacillus rhamnosus* CRL1505 to modulate the respiratory immune response triggered by Respiratory Syncytial Virus (RSV) infection. Infant mice were nasally treated with clodronate-containing liposomes (CLP) during 2 days (days 0 and 1) to induce the depletion of alveolar macrophages. One day after the last CPL administration mice were orally treated with *L. rhamnosus* CRL1505 or *Lactiplantibacillus plantarum* CRL1506 (10^8 cell/mouse/day) during five consecutive days (days 2–6) and then challenged with RSV (day 8). Mice treated with empty liposomes (ELP), CRL1505 or CRL1506 and then challenged with poly(I:C) were used as controls. Two days after the viral challenge, lung RSV titers, lactate dehydrogenase (LDH) activity, albumin concentrations, and the levels of interferon (IFN)-β, IFN-γ, and interleukin (IL)-10 in broncho-alveolar lavages (BAL) were evaluated. The results represent data from three independent experiments. Asterisks indicate significant differences between the respective ELP and CLP groups. Asterisks in black lines indicate significant differences between the indicated groups. *$P < 0.05$, **$P < 0.01$.

the CLP control group (**Figure 1**). Again, mice treated with ELP or CLP and *L. plantarum* CRL1506 showed respiratory cytokines values that were not different from their respective controls (**Figure 1**).

These results suggest that alveolar macrophages are a key immune cell population involved in the differential cytokine induction and in the protection against lung damage induced by orally administered *L. rhamnosus* CRL1505 in the context of TLR3-mediated inflammation.

Effect of Alveolar Macrophages Depletion on the Capacity of *L. rhamnosus* CRL1505 to Modulate the RSV Infection

We further evaluated the impact of alveolar macrophages depletion in the ability of *L. rhamnosus* CRL1505 to improve the resistance against RSV infection. As shown in **Figure 2**, infant mice treated with CLP had similar lung RSV titers than the observed in ELP-treated mice. The *L. rhamnosus* CRL1505 treatment was able to reduce RSV titers in both ELP- and CLP-treated infant mice. However, the lung viral titers in CLP+CRL1505 mice were significantly higher than the found in the ELP+CRL1505 group (**Figure 2**). It was also observed that *L. rhamnosus* CRL1505 was able to significantly reduce BAL albumin and LDH values in ELP-treated infant mice after the infection with RSV (**Figure 2**). In CLP-treated mice, the levels of BAL albumin and LDH were similar to the infant mice in the ELP control group. *L. rhamnosus* CRL1505 was not able to reduce the levels of BAL albumin or LDH in CLP-treated infant mice when compared to the CLP control group (**Figure 2**). Mice treated with ELP or CLP and *L. plantarum* CRL1506 showed lung injury markers values that were not different from their respective controls after the challenge with RSV (**Figure 2**).

The levels of BAL IFN-β, IFN-γ, and IL-10 were also determined after the RSV infection in infant mice treated with liposomes (**Figure 2**). The viral challenge significantly augmented the levels of BAL IFN-β, IFN-γ, and IL-10 in ELP-treated and CLP-treated mice, with no differences between the groups. Similar to our previous results (13), it was observed that *L. rhamnosus* CRL1505 significantly augmented the levels of BAL IFN-β, IFN-γ, and IL-10 after RSV challenge of infant mice treated with ELP. The levels of both BAL IFN-β and IFN-γ in mice treated with CLP+CRL1505 were significantly lower than the found in the ELP+CRL1505 group (**Figure 2**). However, BAL IFN-γ in the CLP+CRL1505 group was significantly higher than CLP controls. In addition, it was observed that in infant mice that received CLP, the treatment with *L. rhamnosus* CRL1505 was unable to modify BAL IL-10 values after RSV infection when compared to the CLP control group (**Figure 2**). Mice treated with ELP or CLP and *L. plantarum* CRL1506 showed respiratory cytokines values that were not different from their respective controls (**Figure 2**).

These RSV challenge experiments suggest that alveolar macrophages are a key immune cell population involved in the protection against virus-induced lung damage as well as in the differential respiratory cytokine profile induced by orally administered *L. rhamnosus* CRL1505.

Effect of *L. rhamnosus* CRL1505 on Alveolar Macrophages Cytokine Profiles in Response to Poly(I:C) or RSV

Taking into consideration that the previous results suggested that the alveolar macrophages would have a relevant role in the immunomodulatory effect of *L. rhamnosus* CRL1505, the changes induced by the strain on alveolar macrophages cytokine profile were then studied. For this purpose, primary cultures of alveolar macrophages from control, *L. rhamnosus* CRL1505- or *L. plantarum* CRL1506-treated infant mice were performed and cells were challenged *in vitro* with poly(I:C) (**Figure 3**), or RSV (**Figure 4**). Basal production of IFN-β, IFN-γ, IL-6, IL-12 as well as the immunoregulatory cytokines IL-10 and IL-27 was detected in alveolar macrophages cultures. Moreover, the basal levels of all the cytokines evaluated were significantly higher in alveolar macrophages cultures obtained from *L. rhamnosus* CRL1505-treated infant mice when compared to controls (**Figure 3**). On the contrary, the levels of all the cytokines evaluated in alveolar macrophages cultures obtained from *L. plantarum* CRL1506-treated infant mice were not different from controls (**Figure 3**).

The challenge with poly(I:C) (**Figure 3**) significantly increased the levels of IFN-β, IFN-γ, IL-6, and IL-12 in control alveolar macrophages cultures as well as in those obtained from CRL1505- or CRL1506-treated infant mice. However, the concentrations of IL-6, IFN-β, and IFN-γ were significantly higher in alveolar macrophages cultures from *L. rhamnosus* CRL1505-treated infant mice when compared to the control group, while IL-12 was not different from control macrophages. In addition, poly(I:C) challenge significantly increased the levels of IL-10 and IL-27 in alveolar macrophages cultures. However, the concentrations of IL-27 were significantly higher in cultures from *L. rhamnosus* CRL1505-treated infant mice when compared to the control group. No differences were observed in IL-10 levels when control macrophages were compared to those obtained from CRL1505-treated infant mice (**Figure 3**). Similarly, the challenge with RSV significantly increased IFN-β, IFN-γ, IL-6, IL-12, IL-10, and IL-27 in all the experimental groups. However, the concentrations of IFN-β, IFN-γ, IL-6, IL-12, and IL-27 were significantly higher in alveolar macrophages cultures from *L. rhamnosus* CRL1505-treated infant mice when compared to the control group. No differences were observed in IL-10 levels when control macrophages were compared to those obtained from CRL1505-treated infant mice (**Figure 4**). The levels of all the cytokines evaluated in alveolar macrophages cultures obtained from *L. plantarum* CRL1506-treated infant mice were not different from controls after poly(I:C) stimulation (**Figure 3**) or RSV challenge (**Figure 4**).

These results indicate that the signals delivered by orally administered *L. rhamnosus* CRL1505 to alveolar macrophages are capable of modulating the cytokine

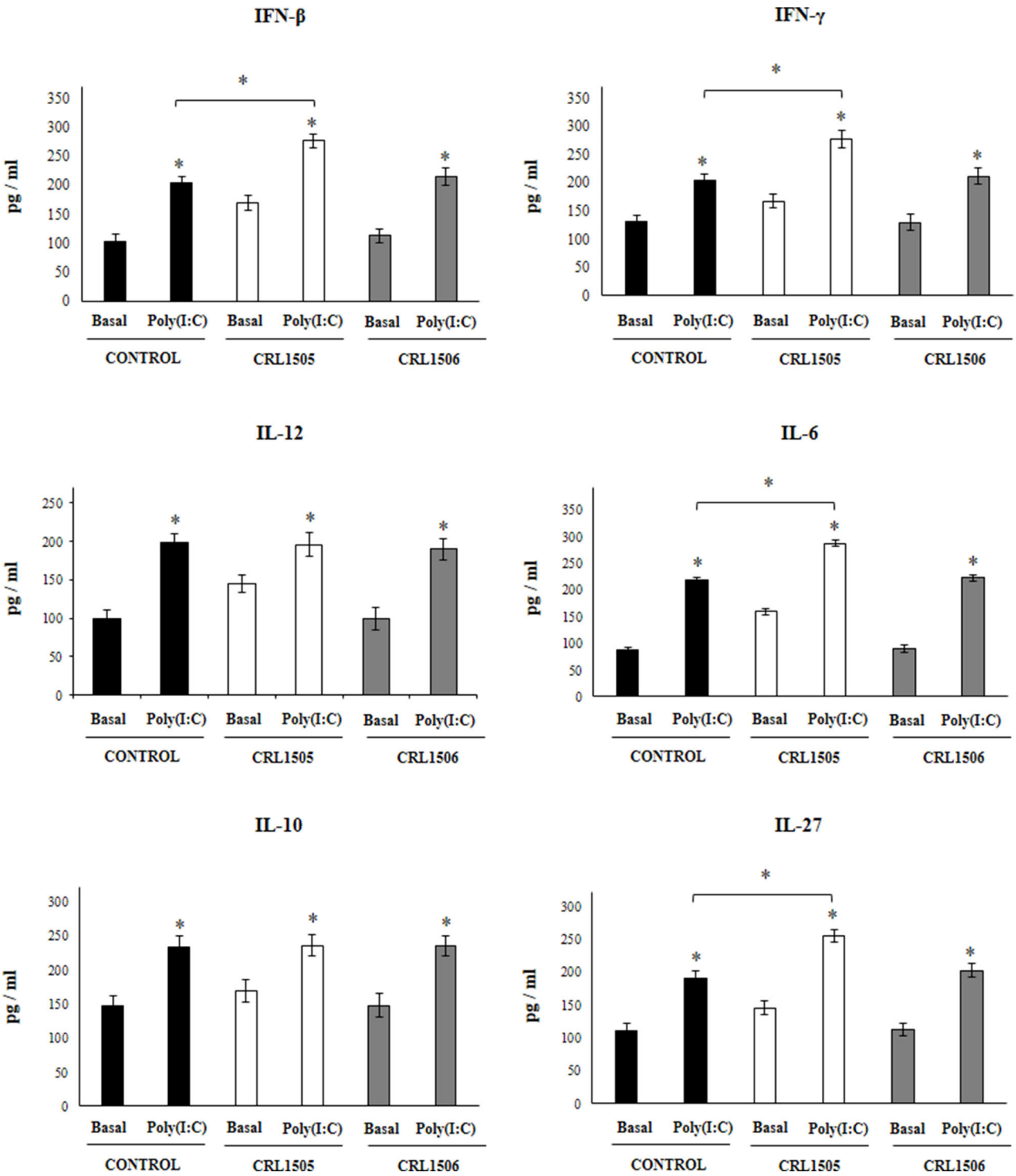

FIGURE 3 | Effect of *Lacticaseibacillus rhamnosus* CRL1505 on alveolar macrophages cytokine profiles in response to poly(I:C) challenge. Infant mice were orally treated with *L. rhamnosus* CRL1505 or *Lactiplantibacillus plantarum* CRL1506 (10^8 cell/mouse/day) during five consecutive days. One day after the last lactobacilli administration, alveolar macrophages were isolated from infant mice, cultured, and challenged *in vitro* with poly(I:C). Twenty-four hours after the poly(I:C) stimulation, the levels of interferon (IFN)-β, IFN-γ, interleukin (IL)-6, IL-10, IL-12, and IL-27 were evaluated on alveolar macrophages supernatants. The results represent data from three independent experiments. Asterisks indicate significant differences between the basal and post-poly(I:C) challenge time points within each group. Asterisks in black lines indicate significant differences between the indicated groups. *$P < 0.05$.

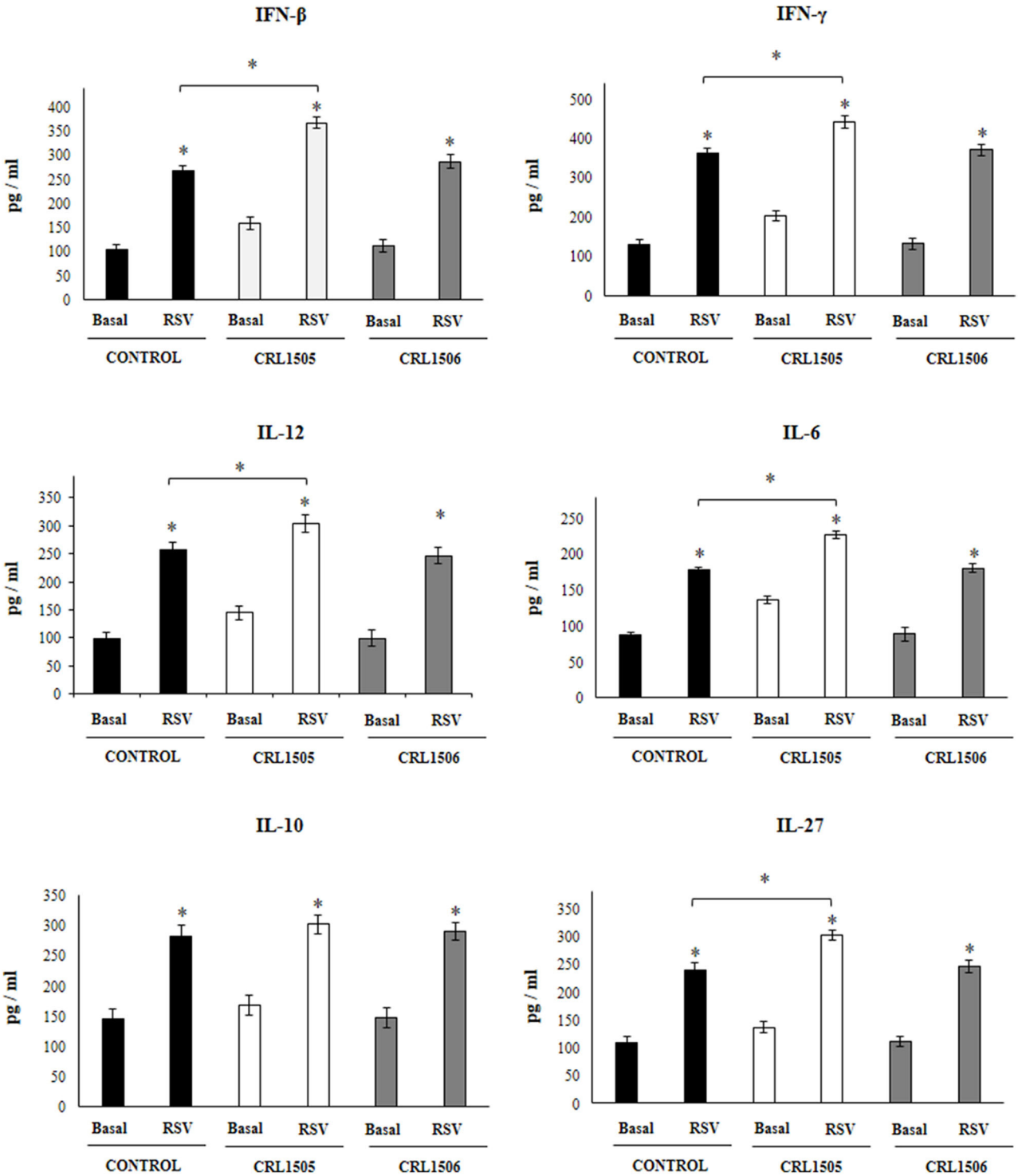

FIGURE 4 | Effect of *Lacticaseibacillus rhamnosus* CRL1505 on alveolar macrophages cytokine profiles in response to Respiratory Syncytial Virus (RSV) infection. Infant mice were orally treated with *L. rhamnosus* CRL1505 or *Lactiplantibacillus plantarum* CRL1506 (10^8 cell/mouse/day) during five consecutive days. One day after the last lactobacilli administration, alveolar macrophages were isolated from infant mice, cultured and challenged *in vitro* with RSV. Twenty-four hours after the viral infection, the levels of interferon (IFN)-β, IFN-γ, interleukin (IL)-6, IL-10, IL-12, and IL-27 were evaluated on alveolar macrophages supernatants. The results represent data from three independent experiments. Asterisks indicate significant differences between the basal and post-RSV challenge time points within each group. Asterisks in black lines indicate significant differences between the indicated groups. *$P < 0.05$.

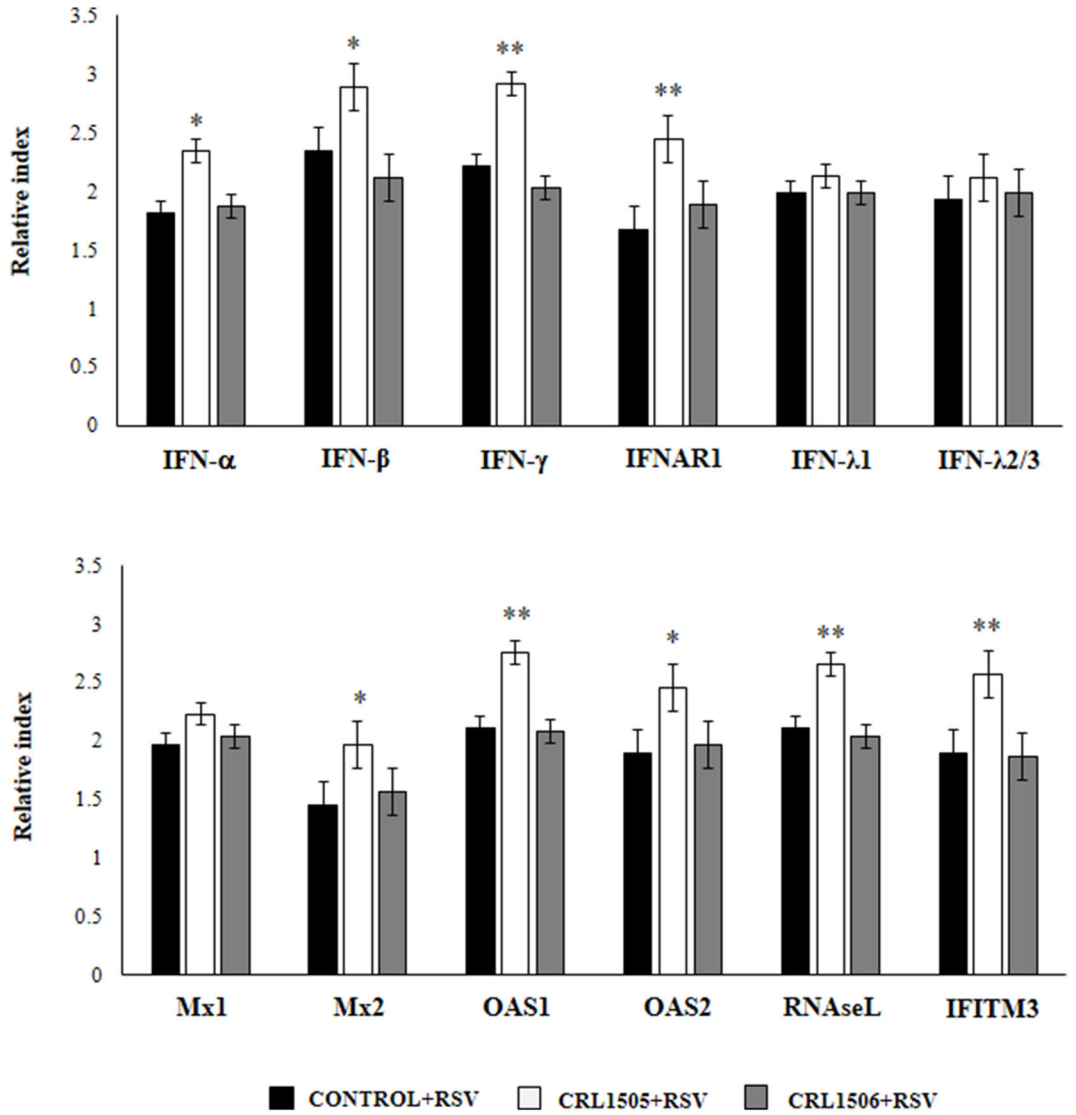

FIGURE 5 | Effect of *Lacticaseibacillus rhamnosus* CRL1505 on alveolar macrophages antiviral factors profiles in response to Respiratory Syncytial Virus (RSV) infection. Infant mice were orally treated with *L. rhamnosus* CRL1505 or *Lactiplantibacillus plantarum* CRL1506 (10^8 cell/mouse/day) during five consecutive days. One day after the last lactobacilli administration, alveolar macrophages were isolated from infant mice, cultured and challenged *in vitro* with RSV. Twelve hours after the viral infection, the expression of *IFN-α, IFN-β, IFN-γ, IFNAR1, IFN-λ1, IFN-λ2/3, Mx1, Mx2, OAS1, OAS2, RNAseL,* and *IFITM3* genes were evaluated by qRT-PCR. The results represent data from three independent experiments. Asterisks indicate significant differences when compared to the control group. $^*P < 0.05$, $^{**}P < 0.01$.

response of these immune cells in front of RSV infection and TLR3 activation.

Effect of *L. rhamnosus* CRL1505 on Alveolar Macrophages Antiviral Factors Expression in Response to RSV Infection

We further characterized the response of alveolar macrophages to RSV challenge by studying the expression levels of *IFN-α, IFN-β, IFN-γ, IFNAR1, IFN-λ1, IFN-λ2/3, Mx1, Mx2, OAS1, OAS2, RNAseL,* and *IFITM3* genes. Primary cultures from alveolar macrophages from control and lactobacilli-treated mice were stimulated with RSV (**Figure 5**). As expected, the expressions of *IFN-α, IFN-β,* and *IFN-γ* in alveolar macrophages from *L. rhamnosus* CRL1505-treated mice were significantly higher than the observed in macrophages from controls or mice treated with *L. plantarum* CRL1506. In accordance with the improved type I IFNs response, the levels of most of the ISGs in macrophages from the CRL1505 group were significantly higher than the control and the CRL1506 groups including *IFNAR1, Mx2, OAS1, OAS2, RNAseL,* and *IFITM3* (**Figure 5**). The only exception was *Mx1,* which expression levels were similar in all the experimental groups. In addition, no differences were found between control and lactobacilli-treated mice when the expression of *IFN-λ1*

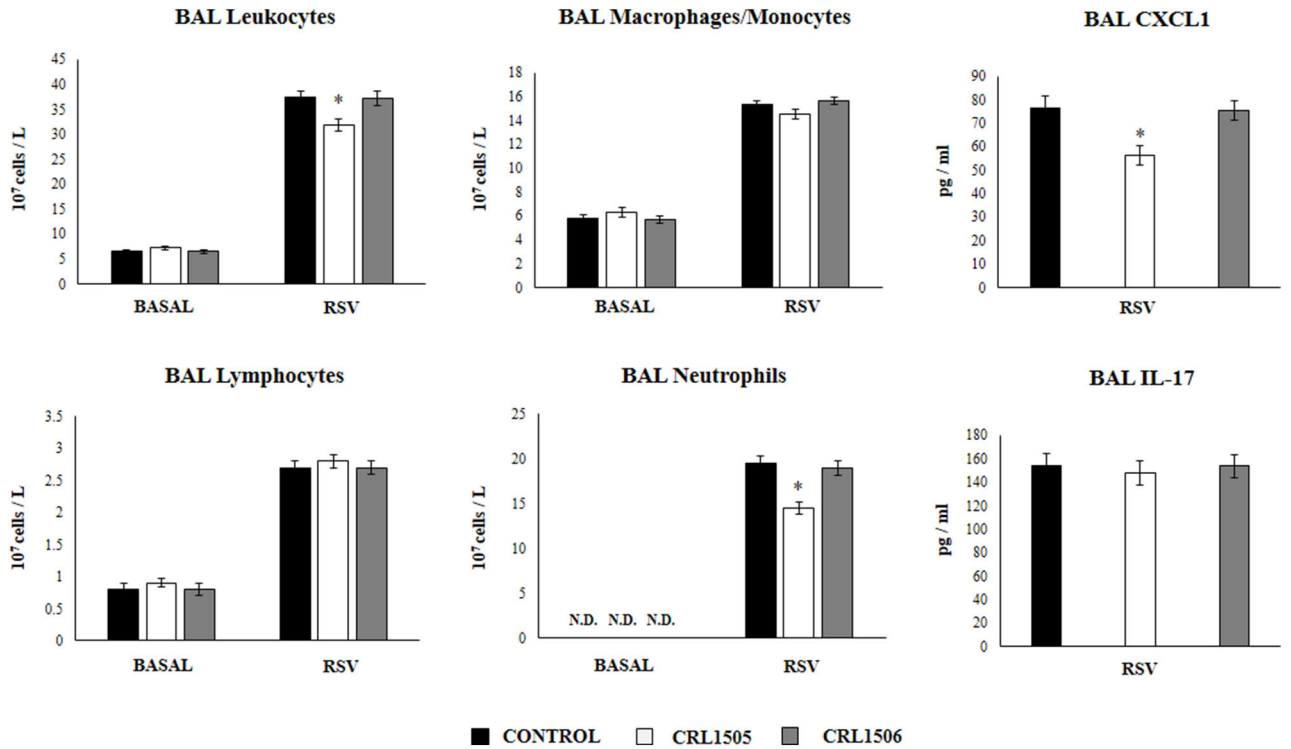

FIGURE 6 | Effect of *Lacticaseibacillus rhamnosus* CRL1505 on broncho-alveolar lavages (BAL) leucocytes variations and CXCL1 and IL-17 levels in response to Respiratory Syncytial Virus (RSV) infection. Infant mice were orally treated with *L. rhamnosus* CRL1505 or *Lactiplantibacillus plantarum* CRL1506 (10^8 cell/mouse/day) during five consecutive days. One day after the last lactobacilli administration, mice were challenged with RSV. The numbers of BAL leucocytes, neutrophils, lymphocytes, and macrophages/monocytes were evaluated before (basal) and 2 days after the RSV infection. The levels of BAL CXCL1 and IL-17 were measured 2 days after RSV challenge. The results represent data from three independent experiments. Asterisks indicate significant differences when compared to the control group. $^*P < 0.05$.

and *IFN-λ2/3* were compared. These results indicate that the signals delivered by orally administered *L. rhamnosus* CRL1505 to alveolar macrophages not only modulate the cytokine response of these immune cells in front of RSV infection but also in addition increase their antiviral state.

Induction of Activated Alveolar Macrophages by *L. rhamnosus* CRL1505

We next aimed to evaluate the influence of the CRL1505 strain on the numbers and activation of immune cell populations in BAL before and after RSV infection. Alveolar macrophages are the main cell population in BAL samples of non-infected mice while lymphocytes are a minor population and neutrophils are not detected (**Figure 6**). *L. rhamnosus* CRL1505 or *L. plantarum* CRL1506 treatments did not modify the numbers of BAL leucocytes in the steady state. The challenge with RSV significantly increased the numbers of all BAL immune cell populations in the three experimental groups. However, CRL1505-treated mice had significantly lower numbers of total leukocytes as well as neutrophils counts when compared to controls (**Figure 6**). In line with this finding, the levels of CXCL1 in BAL samples from CRL1505-treated mice were significantly lower than controls after RSV infection. Of note, BAL IL-17 levels were not different when control and CRL1505-treated animals were compared (**Figure 6**). *L. plantarum* CRL1506 treatment was not able to modify the levels of BAL leukocytes or the concentrations of CXCL1 or IL-17 after the challenge with RSV with respect to the control group.

We further characterized the variations of resident alveolar macrophages in BAL samples by flow cytometry. Total resident alveolar macrophages population in BAL ($CD45^+CD11c^+SiglecF^+$ cells) were evaluated 1 day after lactobacilli treatments, and 2 days after the infection with RSV (**Figure 7**). The total numbers of alveolar macrophages were not modified by lactobacilli treatments in the steady state. The numbers of $CD45^+CD11c^+SiglecF^+$ cell slightly increased upon the challenge with RSV (**Figure 7**) indicating that the increase of total numbers of BAL monocytes/macrophages (**Figure 6**) were mainly produced by recruited cells. No differences were found in $CD45^+CD11c^+SiglecF^+$ cells between lactobacilli-treated and control mice (**Figure 7**). The $CD11c^+SiglecF^+MHC\text{-}II^{hi}$ alveolar macrophages population was also evaluated in BAL samples. In control mice, $CD11c^+SiglecF^+MHC\text{-}II^{hi}$ cells represented around the 25% of the total resident alveolar macrophages population, being most alveolar macrophages

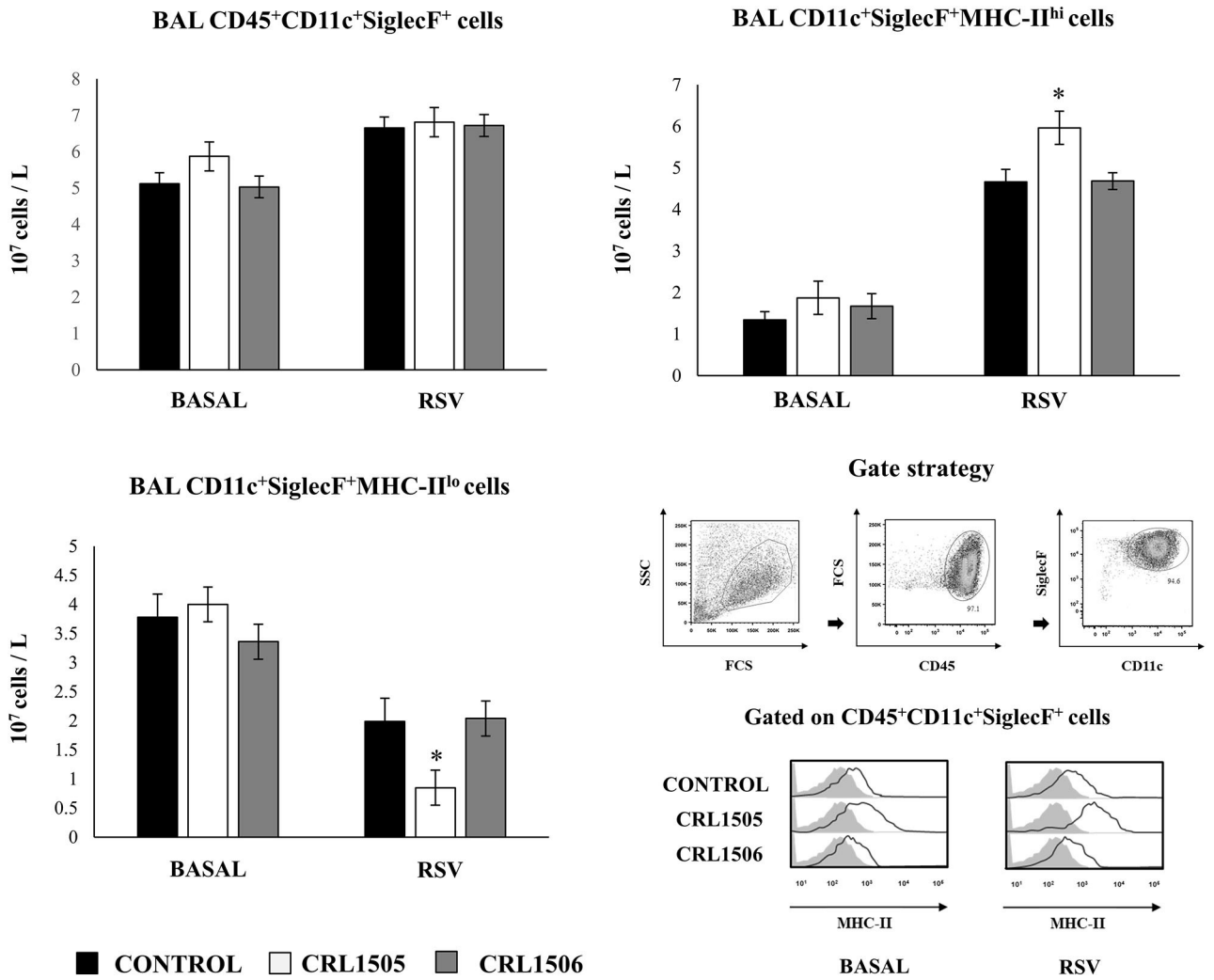

FIGURE 7 | Effect of *Lacticaseibacillus rhamnosus* CRL1505 on resident alveolar macrophages number and activation in response to Respiratory Syncytial Virus (RSV) infection. Infant mice were orally treated with *L. rhamnosus* CRL1505 or *Lactiplantibacillus plantarum* CRL1506 (10^8 cell/mouse/day) during five consecutive days. One day after the last lactobacilli administration, mice were challenged with RSV. Total resident alveolar macrophages populations in broncho-alveolar lavages (BAL) ($CD45^+CD11c^+SiglecF^+$ cells) as well as their expression of MHC-II were evaluated before (basal) and 2 days after the RSV infection. The results represent data from three independent experiments. Asterisks indicate significant differences when compared to the control group. $^*P < 0.05$.

$MHC\text{-}II^{lo}$ cells. The number of $CD11c^+SiglecF^+MHC\text{-}II^{hi}$ cells significantly increased after the RSV infection (**Figure 7**). The treatment with *L. rhamnosus* CRL1505 did not induced statistical significant modifications in the numbers of $CD11c^+SiglecF^+MHC\text{-}II^{hi}$ cells at basal time, although the MFI of MHC-II expression in alveolar macrophages of this group of mice was higher than controls (**Figure 7**). In addition, the numbers of $CD11c^+SiglecF^+MHC\text{-}II^{hi}$ cells in *L. rhamnosus* CRL1505-treated mice were higher than controls after the RSV infection while $CD11c^+SiglecF^+MHC\text{-}II^{lo}$ cells were reduced (**Figure 7**). $MHC\text{-}II^{hi}$ and $MHC\text{-}II^{lo}$ resident alveolar macrophages in mice treated with *L. plantarum* CRL1506 were not different from controls in the two time points evaluated (**Figure 7**). Then, these results allow us to speculate that the *L. rhamnosus* CRL1505 treatment would be able to differentially regulate the recruitment of immune cells into the respiratory tract in response to RSV infection by modulating the activation of alveolar macrophages.

Role of CD4 Cells and IFN-γ in the Modulation of Alveolar Macrophages by *L. rhamnosus* CRL1505

Previously, we demonstrated that the use of anti-IFN-γ blocking antibodies abolish the ability of the CRL1505 strain to reduce RSV titers in infected infant mice. In addition, the blocking of IFN-γ partially affected the reduction of the lung inflammatory damage during the course of RSV infection induced by

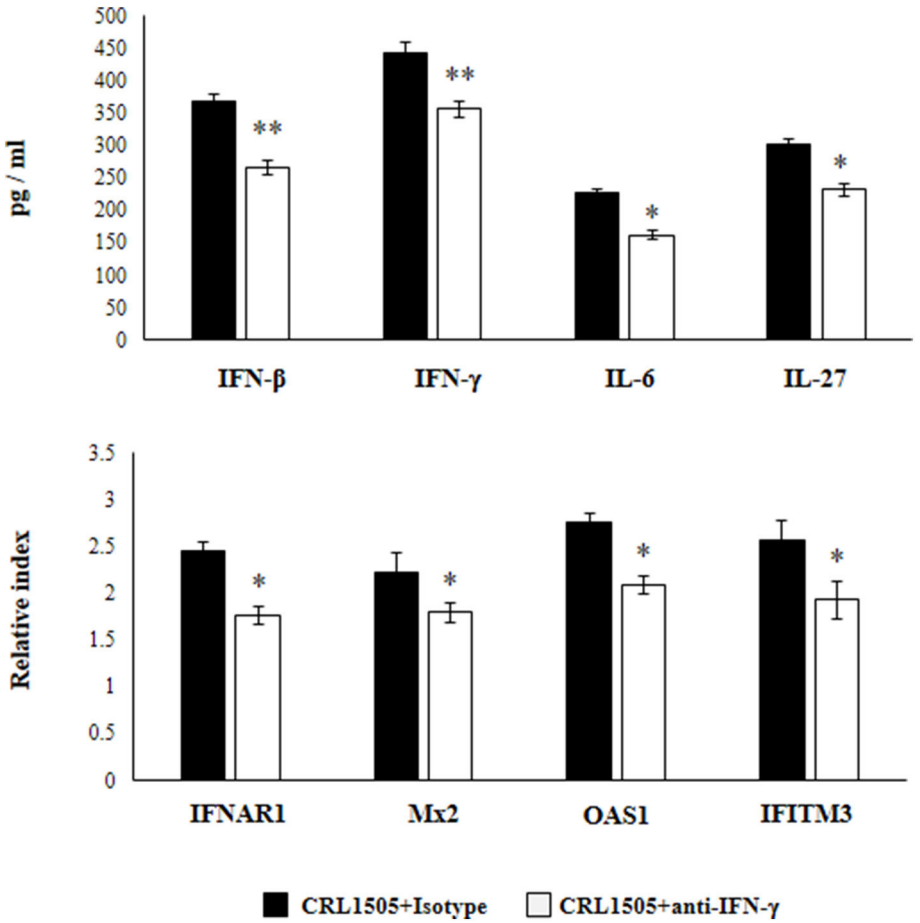

FIGURE 8 | Effect of interferon (IFN)-γ depletion on the ability of *Lacticaseibacillus rhamnosus* CRL1505 to modulate the respiratory immune response triggered by Respiratory Syncytial Virus (RSV) infection. Infant mice were orally treated with *L. rhamnosus* CRL1505 (10^8 cell/mouse/day) during five consecutive days. On days 1, 3, and 5 mice were injected intraperitoneally with purified anti-IFN-γ blocking antibodies or isotype control antibodies. One day after the last lactobacilli administration, alveolar macrophages were isolated from infant mice, cultured, and challenged *in vitro* with RSV. Twelve hours after the viral infection, the expression of *IFNAR1, Mx2, OAS1,* and *IFITM3* genes were evaluated by qRT-PCR. Twenty-four hours after the viral infection, the levels of interferon IFN-β, IFN-γ, interleukin (IL)-6, and IL-27 were evaluated on alveolar macrophages supernatants. The results represent data from three independent experiments. Asterisks indicate significant differences when compared to the isotype control group. $^*P < 0.05$, $^{**}P < 0.01$.

orally administered *L. rhamnosus* CRL1505 (13). Then, we aimed to evaluate whether IFN-γ was related to the effect of the immunobiotic strain on alveolar macrophages. For this purpose, anti-IFN-γ blocking antibodies were administered to mice during the oral treatment with *L. rhamnosus* CRL1505. Primary cultures from alveolar macrophages obtained from these mice and isotype-treated control animals were prepared and stimulated *in vitro* with RSV (**Figure 8**). The production of IFN-β was significantly lower in alveolar macrophages isolated from anti-IFN-γ blocking antibodies-treated mice when compared to isotype controls. Consistently with this finding, it was observed a significant reduction in the expression of *IFNAR1, Mx2, OAS1,* and *IFITM3* genes in alveolar macrophages from anti-IFN-γ blocking antibodies-treated mice than in isotype controls (**Figure 8**). In addition, a significant reduction of IFN-γ, IL-6, and IL-27 production was observed in alveolar macrophages of mice depleted from IFN-γ during *L. rhamnosus* CRL1505 administration (**Figure 8**).

In another set of experiments, anti-CD4 blocking antibodies were administered to mice during the oral treatment with *L. rhamnosus* CRL1505. The response of primary cultures of alveolar macrophages of this group of mice to RSV challenge was also evaluated (**Figure 9**). Similar to IFN-γ blocking experiments, the depletion of CD4 cells during *L. rhamnosus* CRL1505 administration significantly reduced the capacity of alveolar macrophages to produce IFN-β, IFN-γ, IL-6, and IL-27 or express *IFNAR1, Mx2, OAS1,* and *IFITM3* genes in response to RSV challenge (**Figure 9**). Interestingly, anti-CD4 blocking antibodies induced a significantly higher reduction of IFN-β, IFN-γ, and ISGs than anti-IFN-γ blocking antibodies.

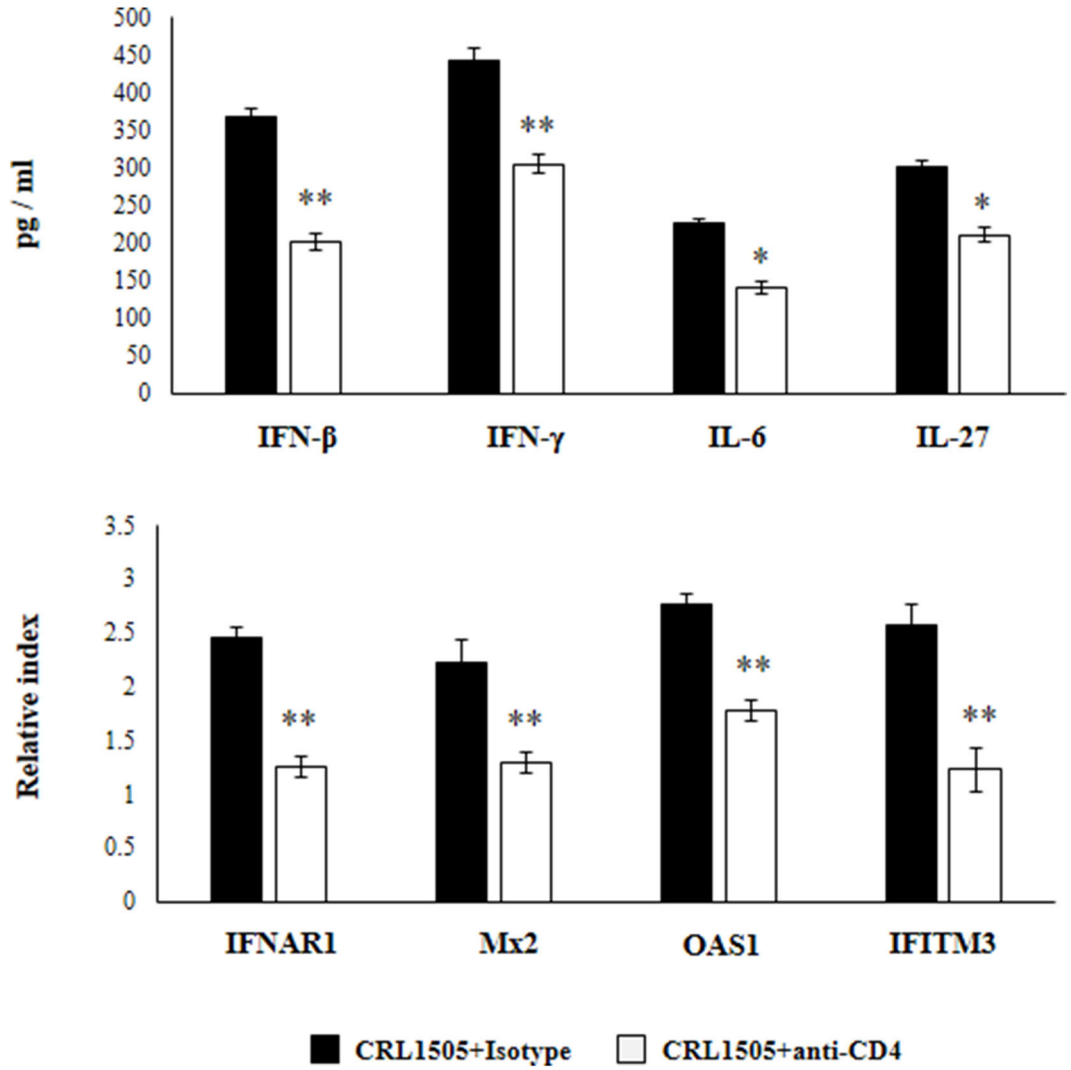

FIGURE 9 | Effect of CD4 T cells depletion on the ability of *Lacticaseibacillus rhamnosus* CRL1505 to modulate the respiratory immune response triggered by Respiratory Syncytial Virus (RSV) infection. Infant mice were orally treated with *L. rhamnosus* CRL1505 (10^8 cell/mouse/day) during five consecutive days. On days 1, 3, and 5 mice were injected intraperitoneally with purified anti-CD4 blocking antibodies or isotype control antibodies. One day after the last lactobacilli administration, alveolar macrophages were isolated from infant mice, cultured, and challenged *in vitro* with RSV. Twelve hours after the viral infection, the expression of *IFNAR1, Mx2, OAS1,* and *IFITM3* genes were evaluated by qRT-PCR. Twenty-four hours after the viral infection, the levels of interferon IFN-β, IFN-γ, interleukin (IL)-6, and IL-27 were evaluated on alveolar macrophages supernatants. The results represent data from three independent experiments. Asterisks indicate significant differences when compared to the isotype control group. $^*P < 0.05$, $^{**}P < 0.01$.

These results highlight the role of IFN-γ and CD4 cells in the modulation of alveolar macrophages activation induced by orally administered *L. rhamnosus* CRL1505.

DISCUSSION

Studies reported an important role for intestinal microbiota in maintaining respiratory antiviral immunity through the modulation of the immune response both at the steady state as well as in response to the viral attack (1–3). Remarkably, research work showed a significant impact of the intestinal microbiota in the respiratory innate antiviral defense mechanisms thought its influence on antigen presenting cells. The studies of Abt et al. (2) demonstrated that the intestinal microbiota help to maintain the optimal functions of pulmonary macrophages. The intestinal microbiota is involved in the efficient capacity of pulmonary macrophages to produce type I IFNs, IFN-λ and antiviral factors including *Irf7*, *Ifngr1*, *Stat1*, *Stat2*, *Mda-5*, *Rig-I*, *Ifit3*, *Mx1*, and *Oas1* to limit IFV replication (2). In line with these findings, some studies reported the ability of orally administered immunobiotic strains to regulate the function of

macrophages in the respiratory tract. It was shown that the oral treatment of adult mice with *L. gasseri* A5 differentially regulated the cytokine profile of alveolar macrophages by increasing their production of IL-12 and decreasing IL-17 and IL-23 (23). The oral administration of a complex mixture of probiotic strains, which included *L. rhamnosus* GG and *E. coli* Nissle 1917 among others, was able to modify the composition of the intestinal microbiota and their metabolic profiles inducing an augmented production of short chain fat acids. These metabolic products influenced alveolar macrophages' function by up-regulating their expression of IFN-β and antiviral factors, increasing the resistance of adult mice to RSV infection (24). In addition, feeding of adult mice with *L. gasseri* SBT2055 improved the expression of *Mx1* and *Oas1* in alveolar macrophages and diminished the susceptibility to IFV infection (25). An enhanced resistance to IFV challenge was also observed in adult mice orally treated with *L. plantarum* DK119. Interestingly, the depletion of alveolar macrophages completely abolished the capacity of the DK119 strain to protect mice against the respiratory viral infection (26).

In line with those studies, we previously reported that orally administered *L. rhamnosus* CRL1505 was capable to modulate alveolar macrophages function in adult mice (27). The CRL1505 strain increased the phagocytic and microbicidal activity of alveolar macrophages, and improved their production of IFN-γ and TNF-α in response to *Streptococcus pneumoniae* challenge. In addition, increased numbers of $CD11c^{+}SiglecF^{+}IFN\text{-}\beta^{+}$ alveolar macrophages were found in mice preventively treated with *L. rhamnosus* CRL1505 and then challenged with poly(I:C) or RSV (13, 14). In this work, we have extended those previous findings by consistently demonstrating a relevant role of alveolar macrophages in the immunomodulatory capacity of orally administered *L. rhamnosus* CRL1505 in the context of RSV infection in infant mice. Macrophages depletion experiments and a detailed study of their production of cytokines and antiviral factors clearly demonstrated the key role of this immune cell population in the improvement of both viral elimination and protection against lung tissue damage induced by the immunobiotic CRL1505 strain.

The phagocytic activity of alveolar macrophages is crucial for the elimination of infected cells during the course of respiratory virus infection. In addition, it was reported that alveolar macrophages play a prominent role in the defense against respiratory viruses such as RSV by producing type I IFNs (28). Moreover, some studies suggested that alveolar macrophages are the most important producers of IFN-α/β in response to RSV challenge, even when compared to other respiratory cells such as epithelial cells and plasmacytoid DCs (29, 30). Type I IFNs produced by alveolar macrophages can act on this same cell population or on other immune and non-immune cells of the respiratory tract modulating their expression of hundreds of ISGs that contribute to viral clearance (31). Type I IFNs production by alveolar macrophages also induce the expression of several monocyte chemoattractants in the respiratory tract that induce the recruitment of $CD11c^{lo}CD64^{hi}CD11b^{+}$ inflammatory monocytes/macrophages that further support the clearance of virus-infected cells (29). On the other hand, it was reported that RSV is capable of infecting both human and murine alveolar macrophages although the infection is abortive since there is no increment of viral particle production (18, 32). However, RSV induce a significant impairment in the production of IFN-γ and IL-12 by alveolar macrophages (33) that was associated with an enhanced severe illness in infants (34). The reduced levels of IFN-γ diminish alveolar macrophages activation, impairing their phagocytic function and their ability to induce the recruitment of T and NK cells to the lungs, contributing to higher viral replication (35, 36). Then, the efficient and timely production of type I IFNs, ISGs and IFN-γ is important to confer protection against RSV.

We previously reported enhanced levels of IFN-β and IFN-γ in BAL samples of CRL1505-treated mice after TLR3 activation (13), RSV challenge (14) or IFV infection (37). The results of this work clearly demonstrated the ability of orally administered *L. rhamnosus* CRL1505 to enhance the ability of alveolar macrophages to produce type I IFNs, ISGs and IFN-γ in response to RSV infection. Our results indicate that alveolar macrophages greatly contribute to the augment of IFN-β and IFN-γ in the respiratory tract of CRL1505-treated mice. Moreover, we reported here for the first time an improved expression of *IFNAR1, Mx2, OAS1, OAS2, RNAseL,* and *IFITM3* in alveolar macrophages after the oral treatment with *L. rhamnosus* CRL1505. OAS1 is capable of inhibiting protein synthesis and viral growth by degrading viral and cellular RNA while IFITM3 has the ability to block early events in the viral replication cycle. Both, OAS1 and IFTIM3 have been shown to interfere with RSV replication and limit productive infection (31, 38). The members of OAS family are able to activate RNAseL, being AOS2 more efficient that OAS1 to induce this effect. The intracellular endoribonuclease RNaseL activated by OAS molecules cleaves viral and cellular RNA resulting in apoptosis (39). It was shown that IFN-γ is able to up-regulate the activities of OAS/RNAseL increasing the protection against RSV infection (40). In addition, recent studies in cotton rats demonstrated differences in RSV infection severity related to the age. While adult animals were resistant to RSV infection, infant rats were highly susceptible to the viral infection. Interestingly, the work reported that the higher viral load and lung pathology observed in younger animals was related to a lower ability to up-regulate IFN-α/Mx2 levels in the respiratory tract (41). The enhancement of these antiviral factors is consistent with the improved clearance of RSV (14) and IFV (37) induced by the oral treatment with the immunobiotic strain. Furthermore, the identification of the differential antiviral factors and cytokines profiles induced by *L. rhamnosus* CRL1505 in alveolar macrophages indicate that the immunobiotic treatment has the potential to protect against other respiratory viruses as well.

The role of alveolar macrophages in the protective immune responses against RSV have been demonstrated in some animal models in which this immune cell population was specifically depleted. Experiments in adult mice demonstrated that the depletion of alveolar macrophages by the administration of

clodronate liposomes before the challenge with RSV significantly impaired the production of IFN-α, TNF-α, and IL-6 in the respiratory tract and diminished the activation and recruitment of NK cells. Those changes were associated to an enhanced lung RSV load (42). Experiments in CD169-diphtheria toxin receptor transgenic mice, which are depleted from $CD169^+$ alveolar macrophages after the administration diphtheria toxin, also demonstrated that macrophages elimination impaired the production of IFN-β, IL-6, and TNF-α in the respiratory tract in response to RSV infection (43). By using a similar approach, we demonstrated here that the depletion of alveolar macrophages by the time of *L. rhamnosus* CRL1505 administration abolished the ability of infant mice to produce improved levels of IFN-β in response to TLR3 activation or RSV infection. In addition, although the production of IFN-γ was diminished when alveolar macrophages were depleted; the levels of this cytokine were still significantly higher in CRL1505-treated mice than in controls. These results indicate that other immune cell population also contribute to the improved levels of IFN-γ in the respiratory tract of CRL1505-treated mice. The most likely source of IFN-γ are CD4+ T cells as discussed below. Of note, the depletion of alveolar macrophages completely abolished the ability of orally administered *L. rhamnosus* CRL1505 to improve IL-10 in the respiratory tract or to reduce the biochemical markers of lung injury after TLR3 activation or RSV infection. Then, alveolar macrophages had a key role in the protection against the lung detrimental inflammation induced by the immunobiotic CRL1505 strain.

The alteration of alveolar macrophages function by the infection with RSV has been associated to an exacerbated viral-mediated bronchiolitis (35). In fact, the depletion of alveolar macrophages greatly increased the recruitment of inflammatory cells to the lung during the early stage of RSV infection. The depletion of alveolar macrophages induce a significant greater lung inflammation in response to RSV challenge that is characterized by increases in $CD11b^{hi}Gr1^{hi}$ neutrophils and inflammatory $CD11c^{hi}MHC\text{-}II^{hi}CD11b^+$ DCs that contribute to an hyperresponsiveness in RSV-infected mice (18). Notably, this deregulated inflammatory response contribute poorly to the elimination of the virus while promoting local damage and affecting lung function. Then, the immunoregulatory functions of alveolar macrophages seems crucial for avoiding the lung inflammatory-mediated damage during the course of RSV infection. Several mechanisms have been proposed for the immunoregulatory functions of alveolar macrophages in the context of viral infections. The most obvious function of macrophages is the phagocytosis of virus-infected apoptotic cells preventing the release of cellular contents to local environment and avoiding the triggering of further inflammatory factors production (44). It was also shown that alveolar macrophages produce anti-inflammatory cytokines such as IL-10, especially during the resolution of the infection (32, 44). Another anti-inflammatory strategy of alveolar macrophages is their ability to promote Treg cells responses by directly interacting with these cells or indirectly through the production of certain cytokines (45, 46). It was reported that resident $CD11c^+SiglecF^+$ alveolar macrophages pulsed *in vitro* with ovalbumin are able to induce the development of $Foxp3^+$ Treg cells when they are co-cultured with antigen-specific CD4 T cells (45). Moreover, the transfer of the ovalbumin-pulsed macrophages into the respiratory tract of mice significantly reduced the lung inflammation upon subsequent stimulation with the antigen. On the other hand, it was demonstrated that IL-6 is required for the reduction of RSV-induced immunopathology. The early production of IL-6 after RSV challenge stimulates the expression of IL-27 by alveolar macrophages, which in turn promotes the maturation of Treg cells in the respiratory tract (46). Our previous studies (13, 14, 37) and the results presented here allow us to conclude that some of these anti-inflammatory functions of alveolar macrophages are enhanced by the oral administration of *L. rhamnosus* CRL1505.

We have reported increased levels of IL-10 in the respiratory tract of *L. rhamnosus* CRL1505-treated mice and partially attributed this increment to the enhanced production of this immunoregulatory cytokine by alveolar macrophages (13, 14, 27). The data of this work show that this assertion was incorrect, since the study of the production of IL-10 by the alveolar macrophages in CRL1505-treated infant mice were not different from controls at the basal level or upon stimulation with poly(I:C) or RSV. On the other hand, we demonstrated here for the first time that alveolar macrophages from *L. rhamnosus* CRL1505-treated mice had a significantly increased capacity to produce IL-27 in response to TRL3 activation or RSV stimulation. The immunoregulatory cytokine IL-27 have been shown to modulate inflammatory responses by acting in cells from both innate and adaptive immunity (47). Recent studies highlighted the role of IL-27 in the protection against lung inflammatory damage during the course of viral infections. It was reported that $IL\text{-}27RA^{-/-}$ mice have an exaggerated lung immunopathology after the infection with IFV, that correlated with increased levels of $CD4^+$ and $CD8^+$ T cells producing IL-17 and a strong neutrophil infiltration (48). In addition, the depletion of IL-27 in the respiratory tract during RSV infection enhanced the damaging inflammation (46). It was also shown that IL-27 helps in the control of RSV infection severity by suppressing Th17- and Th2-mediated inflammation (49). Interestingly, comparative studies of IFV infection in $IL\text{-}27RA^{-/-}$ and $IL\text{-}10^{-/-}$ mice demonstrated that the former had a more severe disease course than the latter (48), demonstrating that not all the anti-inflammatory effects of IL-27 are mediated by the induction of IL-10 production as it has been suggested (47, 50, 51). Of note, it was reported that IL-27 is not sufficient for the optimal induction of Treg cells maturation in the respiratory tract and that IL-6 is required for the IL-27/Treg cells protection against inflammatory damage. The early production of IL-6 after RSV infection induce the production of IL-27 by myeloid cells including alveolar macrophages, which in turn stimulates Treg cell maturation. Depletion of IL-27 or IL-6 in the respiratory tract during RSV have the same detrimental effect on the maturation of Treg cells and RSV-mediated immunopathology (46). Then, our results show that the improved production of IL-27 and IL-6 by alveolar macrophages of CRL1505-treated mice may play an important role in

limiting inflammation and protecting lung function during RSV infection, by increasing the maturation and activation of Treg cells.

Macrophage's functions have been studied in the context the M1/M2 polarization dichotomy being classically activated macrophages (M1-like) associated to pro-inflammatory microenvironments while alternatively activated macrophages (M2-like) developed in anti-inflammatory environments (52). M1-like macrophages are characterized by a high phagocytic activity and by their ability to guide acute inflammatory responses through their production of pro-inflammatory factors that stimulate the Th1 response. On the other hand, M2-like macrophages negatively regulate pro-inflammatory cytokines, and induce the production of anti-inflammatory mediators contributing to the control of inflammation, tissue repair and the return to hemostasis of the infected tissue (52). However, recent advances in the biology of macrophages have demonstrated that this dichotomy separating M1-like and M2-like macrophages is rather represented by a continuum between the two states. This fact is particularly true for alveolar macrophages that have been shown to express a combined M1/M2 phenotype (53). It was suggested that this hybrid phenotype confer to alveolar macrophages the ability to quickly switch between M1 or M2 associated functions allowing for appropriate responses to stimuli and tissue environment. During the acute phase of inflammation, alveolar macrophages may function as M1 activated cells inducing the activation of various mechanisms that contribute to pathogen elimination. However, such responses must be controlled to prevent lung tissue damage through the activation of M2 functions that control inflammatory reactions, and promote and accelerate the wound healing process and tissue repair. Such a fine-tuned balance and switching back and forth between the M1 and M2 polarization states in alveolar macrophages would be necessary to allow the beneficial processes of inflammation, resolution, and repair (52, 53). Then, it could be concluded that the oral treatment with *L. rhamnosus* CRL1505 would not induce differential modifications of alveolar macrophages but instead it would stimulate and improve a function that is already programmed in this particular population of immune cells of the respiratory tract.

As mentioned before, studies demonstrated that alveolar macrophages have a great impact in the innate antiviral immune response to RSV. However, their role in the generation of adaptive immune responses has not been completely elucidated. It was shown the macrophage depletion did not affect the recruitment of activated $CD4^+$ T cells into the lung, indicating that this immune cell population may have little effect on the adaptive response to RSV (42). In contrast, the depletion of alveolar macrophages before the nasal immunization with an experimental RSV vaccine based on the viral G protein significantly reduced the levels of specific neutralizing antibodies (54). Mice with depleted alveolar macrophages had an impaired protection against RSV challenge when compared to normal controls. In addition, the depletion of $CD169^+$ alveolar macrophages reduced the recruitment of effector $CD8^+$ T cells to the lungs after RSV infection (43). On the other hand, it was reported that the influence of intestinal microbiota on the respiratory innate antiviral immune response is able to condition the subsequent adaptive immune responses. The intestinal microbiota was shown to positively influence the ability of pulmonary macrophages to support the generation of virus-specific antibodies as well as virus-specific T cells. In intestinal microbiota-depleted mice, macrophages had a reduced expression of MHC-I and CD86 molecules. An impaired number of IFV-specific $CD8^+$ T cells as well as a reduced ability of these cells to produce IFN-γ, TNF-α, IL-2, and MIP-1α was also observed (2). Taking into consideration the findings of this work demonstrating that orally administered *L. rhamnosus* CRL1505 influence the MHC-II expression of the alveolar macrophages during the course of RSV infection, it is tempting to speculate that the immunobiotic intervention would also beneficially modify the adaptive immune response against the viral pathogen in infant mice. In support of this hypothesis, it has been shown that IFN-γ is able to up-regulate MHC-II expression in alveolar macrophages improving their antigen presentation activities (55). The evaluation of the influence of *L. rhamnosus* CRL1505 in the adaptive immune response to RSV and the role of alveolar macrophages in this potential beneficial effect is an interesting topic for future research.

In addition, we provided evidence that support an important role of $CD4^+$ cells and IFN-γ in the ability of *L. rhamnosus* CRL1505 to modulate alveolar macrophages activities and the subsequent improved response to RSV infection. In our hands, the administration of anti-IFN-γ or anti-CD4 antibodies at the time of the CRL1505 treatment completely abolished the ability of the immunobiotic strain to differentially modulate the cytokine and antiviral factors profile of alveolar macrophages. The inefficient production of IFN-γ in the respiratory tract during the course of RSV infection with the subsequent impaired activation of alveolar macrophages has been associated with an increase of severe bronchiolitis and pneumonia in neonates and infants (34–36). In addition, comparative studies of RSV challenge in infant and adult mice demonstrated that in IFN-γ production were associated to the distinct susceptibility of both groups to viral infection (36, 55). Adult mice are able to induce the recruitment of $CD8^+$IFN-γ^+ and $CD4^+$IFN-γ^+ T cells in the lung, which activated alveolar macrophages and promoted an efficient viral clearance. In contrast, infant mice were capable of inducing the recruitment of $CD8^+$IFN-γ^+ T cells in the respiratory tract. The lack of effective recruitment of $CD4^+$IFN-γ^+ T cells into the alveolar space and the lower activation of macrophages correlated with the higher RSV loads in the lungs of infant mice. These previous findings and the results of this work highlight the role of $CD4^+$ cells and IFN-γ in the activation of alveolar macrophages induced by *L. rhamnosus* CRL1505 and in the modulation of respiratory antiviral immunity.

The exact origin and nature of the signals used by the intestinal microbiota or orally administered immunobiotics to modulate the respiratory antiviral immunity remain to be determined. Our experiments blocking $CD4^+$ cells and IFN-γ allow us to hypothesize about a potential mechanism that could explain

the remote effect induced by orally administered *L. rhamnosus* CRL1505. The existence of the so-called common mucosal immune system implies that the immune cells activated in one mucosal tissue can mobilize and reach distant mucosal sites where they can influence immune responses. Then, the mobilization of B and T cells from the intestinal mucosa to the respiratory tract could be involved in the beneficial effects exerted by the intestinal microbiota or immunobiotics (6, 56). It was also hypothesized that immune factors such as cytokines and growth factors produced in the intestinal mucosa in response to microbiota stimulation, can be released to blood and act systemically or in other mucosal tissues (56, 57). We previously demonstrated that both orally administered *L. rhamnosus* CRL1505 and *L. plantarum* CRL1506 were capable of improving the levels of IFN-γ in the intestine and blood, while only the CRL1505 strain increased this immune factor in the respiratory tract, indicating that the IFN-γ was produced locally (13). In addition, our previous results indicated that the oral administration of *L. rhamnosus* CRL1505 induces an increase of $CD4^+$IFN-γ^+ T cells in the lungs, and effect that was not observed in CRL1506-treated mice (14). Then, considering those previous results and the ones obtained in this work it is tempting to speculate that *L. rhamnosus* CRL1505 would induce the mobilization $CD4^+$IFN-γ^+ T cells from the intestine to the lungs, and that the IFN-γ produced by mobilized $CD4^+$ T cells would modulate the respiratory tract innate immune microenvironment leading to the activation of local immune cells such as alveolar macrophages. Conducting more in-depth studies to verify this hypothesis is a task that we intend to carry out in the immediate future.

Of note, other probable mechanisms have been proposed, which are not mutually exclusive, to explain the effect of the intestinal beneficial microbes on the respiratory antiviral immunity. There is evidence that some microbial-associated molecular patterns (MAMPs) derived from the intestinal microbiota can be adsorbed and transported to extraintestinal sites where they stimulate pattern recognition receptors (PRRs) expressed in non-immune and immune cells influencing the immune responses (58, 59). In addition, microbial metabolites that are adsorbed in the intestine have been associated to the differential modulation of respiratory immune responses. This effect has been called "metabolic reprograming" (60) and implies that metabolites such as circulating short-chain fatty acids (61), desaminotyrosine (5), docosahexanoic acid (62), or acetate (4) can reach innate immune cells in the respiratory tract and improve their responses to viral infections. Studying whether these mechanisms contribute to the immunomodulatory effect of *L. rhamnosus* CRL1505 is also an interesting topic for future research.

Alveolar macrophages are a crucial component of innate host defense in the respiratory tract and this immune cell population have been demonstrated to play a critical role limiting the severity of RSV-induced disease. In this study, we defined the importance of alveolar macrophages as a key players in the immunomodulatory and protective activities of orally administered *L. rhamnosus* CRL1505 in the context of RSV infection in infants. In addition, we provided evidence of the important role of $CD4^+$ cells and IFN-γ in the activation of alveolar macrophages highlighting a putative pathway through which the intestinal and respiratory mucosa are communicated under the influence of *L. rhamnosus* CRL1505.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Guidelines for Animal Experimentation of CERELA. The CERELA Institutional Animal Care and Use Committee prospectively approved this research.

AUTHOR CONTRIBUTIONS

JV and HK designed the study and wrote the manuscript. VG-C, MT, FR, and MI did the laboratory work. VG-C and MI performed statistical analysis. HT, HK, and JV contributed to data analysis and interpretation. All authors read and approved 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.2020.568636/full#supplementary-material

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REVIEW
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Paraprobiotics and Postbiotics of Probiotic *Lactobacilli,* Their Positive Effects on the Host and Action Mechanisms: A Review

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Lactobacilli comprise an important group of probiotics for both human and animals. The emerging concern regarding safety problems associated with live microbial cells is enhancing the interest in using cell components and metabolites derived from probiotic strains. Here, we define cell structural components and metabolites of probiotic bacteria as paraprobiotics and postbiotics, respectively. Paraprobiotics and postbiotics produced from *Lactobacilli* consist of a wide range of molecules including peptidoglycans, surface proteins, cell wall polysaccharides, secreted proteins, bacteriocins, and organic acids, which mediate positive effect on the host, such as immunomodulatory, anti-tumor, antimicrobial, and barrier-preservation effects. In this review, we systematically summarize the paraprobiotics and postbiotics derived from *Lactobacilli* and their beneficial functions. We also discuss the mechanisms underlying their beneficial effects on the host, and their interaction with the host cells. This review may boost our understanding on the benefits and molecular mechanisms associated with paraprobiotics and probiotics from *Lactobacilli*, which may promote their applications in humans and animals.

Keywords: paraprobiotics, postbiotics, *Lactobacilli*, metabolites, immunomodulatory effect

INTRODUCTION

The genus *Lactobacillus* is the largest genus among lactic acid bacteria (LAB), consisting of more than 237 species (1), with continuous new species discoveries, such as *Lactobacillus metriopterae* (2) and *Lactobacillus timonensis* (3). Some *Lactobacillus* species are among the most widely used probiotics (4). Accumulating evidences are proposing that probiotic cell components or metabolites which interacting with the host cells may trigger probiotic effects (5–9). The advantages of metabolites and cell components of these probiotic bacteria over probiotic bacteria were clarified (10, 11). Furthermore, it has been reported that not

all probiotic bacteria are safe. Concerns associated with live probiotic bacteria administration have been described in case reports, clinical trials and experimental models (12–14). Therefore, the applications of cell components or metabolites derived from probiotic strains are gaining more interest.

Regarding the use of cell components and metabolites of probiotics, different terms have been proposed, such as "paraprobiotics," "ghost probiotics" "inactivated probiotics" "non-viable microbial cells," "metabolic probiotics" "postbiotics," etc. The concept of paraprobiotics was proposed to indicate the use of inactivated microbial cells or cell fractions that confer health benefit to the host (15). In some studies, cell wall components of the probiotics are categorized as paraprobiotics (16). Postbiotics are defined as soluble products or metabolites secreted by probiotics that have physiological benefits to the host (9). Similar definition as "factors resulting from the metabolic activity of a probiotic or any released molecules capable of conferring beneficial effects to the host in a direct or indirect way" was made by other researchers (17). To better differentiate cellular structural components and metabolites of probiotic strains, we define the cell structural components (mainly cell wall components) as paraprobiotics and secretory metabolites/componnets as postbiotics in this review.

The potential health benefits of probiotic *Lactobacillus* species isolated from the intestine of humans and animals have been documented in a plethora of research publications to date. The terms of paraprobiotics and postbiotics have emerged recently, but they have been adopted rapidly in several study areas including food science, food microbiology, and health and nutrition of human and animals. However, knowledge on the types of paraprobiotics and postbiotics is limited and some aspects related to the bioactivities and the action mechanisms of health-promoting effects of paraprobiotics and postbiotics remain unclear. The present review aims to update the evidence on the paraprobiotics and postbiotics derived from *Lactobacilli*, their physiological benefits and mechanism of interaction with the host cells.

ISOLATION AND PURIFICATION OF PARAPROBIOTICS AND POSTBIOTICS

Scientific evidences showed that there are different methods to isolate and purify paraprobiotics and postbiotics from several *Lactobacilli* species. Isolation of paraprobiotics and postbiotics from different probiotic bacteria involve cell disruption techniques including thermal treatment (18, 19), enzymatic treatments (60), solvent extraction (20), radiation (ionizing and UV rays) (21), high pressure (22) and sonication (23–26). Several other methods also have the potential to be used for production of paraprobiotics and postbiotics, such as ohmic heating and supercritical CO_2, drying, pulsed electric field (PEF), and pH changes (27).

During the production of paraprobiotics from probiotics, it is important to expose the cells to factors (27) without disrupting cell structure (9). On the other hand, to isolate intracellular postbiotics, it is required to disrupt the bacterial membrane via combined treatments in order to obtain the intracellular metabolites (9). Furthermore, extraction and clean-up steps have been applied to help the isolation procedures, such as centrifugation, dialysis, lyophilization and column purification (23, 28–30). Secreted postbiotics by viable cells can be recovered from supernatants, and the viable cells can be eliminated from the medium by centrifugation and/or filtration (31). In most of the time, we can isolate paraprobiotics and postbiotics. However, in some cases it is difficult to separate them, and additional steps such as microfiltration are necessary to isolate the postbiotic fraction. The choice of techniques for isolation of postbiotics and paraprobiotics depend on the characteristics of molecules under study (32). Since the health benefits of paraprobiotics and postbiotics are influenced by their isolation methods, it is important to select the best methods and conditions for probiotic inactivation to obtain paraprobiotics and postbiotics (33).

CATEGORIES, PROPERTIES, AND POSITIVE EFFECTS OF PARAPROBIOTICS AND POSTBIOTICS DERIVED FROM *LACTOBACILLUS*

Studies described that most of the paraprobiotics are located in the bacterial cell-envelope (5, 34). Generally, paraprobiotics consist of a wide range of molecules including peptidoglycans, surface proteins, cell wall polysaccharides, while postbiotics include secreted proteins and peptides, bacteriocins, organic acids, etc (10, 35–37). Furthermore, the paraprobiotics and postbiotics mediate a wide range of positive effects on the host such as immunomodulatory, anti-tumor, barrier-preservation, and antimicrobial properties (24, 38). Different species of *Lactobacillus* have different types of paraprobiotics and postbiotics. In the following part, we summarized the chemical composition and beneficial functions of paraprobiotics and postbiotics derived from *Lactobacilli* (**Table 1**).

Paraprobiotics

Studies confirmed that cell surface components of *Lactobacilli* are considered as an important part of effector molecules, as this part of the microbial cell is the first to interact with host cells. The cell envelope components of *Lactobacilli,* here categorized as paraprobiotics, include peptidoglycan, teichoic acid, cell-wall polysaccharides, cell surface-associated proteins, and proteinaceous filaments, which have been reported to mediate beneficial effects to the host (**Figure 1**).

Peptidoglycan

The cell wall of *Lactobacilli* contains a thick peptidoglycan layer, which is a multilayer, cross-linked glycan chain with a repeating pentapeptide unit of β-1,4-linked *N*-acetylglucosamine and *N*-acetylmuramic disaccharide units (107) and the fundamental composition of the glycan strands and pentapeptides was strain-specific for *Lactobacilli* (108). At the time of biosynthesis, assembly, and incorporation of peptidoglycan components, modifications happen in the bacterial peptidoglycan which could

TABLE 1 | Probiotic effects of paraprobiotics and postbiotics in *Lactobacilli*.

Probiotic effects	Paraprobiotics/ postbiotics	Model	References
Immunomodulatory effects	Peptidoglycan	Mice	(39)
	Teichoic acid	Porcine intestinal epithelial cell line	(7)
	Cell-wall polysaccharides	Mice and human cell lines	(40–42) (8, 43)
	Exopolysaccharides	Human cell lines	(39, 44, 45)
	S-layer proteins	Mice, 3T3 cells, Mouse cells	
	Pili proteins	Human cell, Murine cell lines	(46–48)
	Oligodeoxynucleotide (ODN)	Human cell lines	(49)
	Pyroglutamic acid dipeptides	Mouse cell lines	(50)
	Serine-Threonine peptide	Human cell lines	(51)
	Bacteriocins	Human and Mouse cell lines	(52–54)
	Short chain fatty acids	Mouse cell lines	(55); (56)
	Trp-Indole derivatives	Mouse cell	(57, 58)
	Conjugated linoleic acids	Human epithelium cell lines	(59)
Antagonistic effects against pathogens	Cyclic dipeptides	Human cells	(60)
	Bacteriocins	Human, Mouse cells	(61); (62)
	Conjugated linoleic acids	Human epithelium cell lines	(59)
Anti-tumor effects	Exopolysaccharides	Human colon cancer HT-29 cell	(63, 64)
	Conjugated linoleic acid	Human epithelium cell lines, human prostate cancer cell line	(65); (66)
	S-layer proteins	Human HT-29 cell line	(67); (68)
Preservation of intestinal barrier	LPXTG proteins	Human HT-29 cell line	(69)
	S-layer proteins	Human HT-29 cell line	(70)
	Moonlighting proteins	Human intestinal cell lines	(71)
	Pili proteins	Caco-2 cell line	(46, 72)
	Aggregation-promoting factor	Caco-2 epithelial cell lines	(73)
	p40 and p75 proteins	Mouse cell lines	(74, 75)

enhance the sensitivity to autolysis, hydrophobicity of the cell envelope, and resistance to lysozyme (109).

Peptidoglycan of *Lactobacillus casei* (*L. casei*), *Lactobacillus johnsonii* (*L. johnsonii*) JCM 2012 and *Lactobacillus plantarum* ATCC 14917 was reported to suppress interleukin-12 (IL-12) production via Toll-like receptor 2 (TLR2) which have been associated with autoimmune and inflammatory bowel diseases (94). Purified peptidoglycan from *Lactobacillus salivarius* (*L. salivarius*) Ls33 also exerted anti-inflammatory properties by inducing IL-10 production. Moreover, Ls33 peptidoglycan stimulated dendritic cell and T-cell regulatory functions upon sensing of nucleotide-binding oligomerization domain protein 2 (NOD2), and rescued mice from colitis induced by trinitrobenzene sulfonic acid (TNBS) (95). Furthermore, peptidoglycan from *Lactobacillus rhamnosus* (*L. rhamnosus*) CRL1505 was able to improve innate and systemic adaptive immune responses in mice (39). Notably, strain- or species-specific modifications of the conserved peptidoglycan polymers, including amidation, acetylation, and glycosylation, can lead to specific immunomodulatory capacities, which may contribute to the strain-specificity of probiotic effect.

Teichoic Acid

Teichoic acids (TAs) are the second main constituent of cell walls of *Lactobacilli* and account for up to half of the cell wall dry weight (110). Due to the anionic polymers nature of the TA, it can be covalently linked to peptidoglycan as wall teichoic acid (WTA) or anchored to the cytoplasmic membrane by their lipid anchors as lipoteichoic acid (LTA) (111).

Plethora studies reported the immunomodulatory characteristics of TA from many species of *Lactobacillus* (112). *L. plantarum* LTA (Lp.LTA) attenuated the expression of IL-8 induced by Pam2CSK and exerted anti-inflammatory effects on human intestinal epithelial cells (92). LTA of *L. plantarum* also showed anti-inflammatory responses in porcine intestinal epithelial cells (7). The anti-inflammatory functions and effects of LTAs are species or strain-specific. For instance, it has been shown that the majority of immunomodulatory properties induced by *L. plantarum* TA were dependent on D-alanylation (93).

Cell-Wall Polysaccharides

Polysaccharides are common in gram-positive bacteria surface including *Lactobacilli*. The most studied polysaccharides are exopolysaccharides (EPS). EPS may facilitate the interaction of the bacteria with the environment, mediate adhesion properties, protect against pathogens, and also act as a protective layer (43, 113).

Studies revealed that EPS derived from several species of *Lactobacillus* has a capacity to modulate systemic and mucosal immune responses, and provide direct health-promoting benefits. Purified EPS produced by *L. rhamnosus* RW-9595M exhibited immuno-suppressive effect on macrophages by inducing high levels of IL-10 and low or no tumor necrosis factor alpha (TNF-α), IL-6, and IL-12 (99). Moreover, the EPS-producing *L. plantarum* BGCG11 strain showed anti-inflammatory effect, pointing to an immune-suppressive role of EPS (100). Acidic fraction of EPS produced by *L. plantarum* 14 was able to decrease the production of pro-inflammatory cytokines (IL-6, IL-8, and MCP-1) in porcine intestinal epithelial cells in response to enterotoxigenic *Escherichia coli* (*E. coli*) (ETEC) challenge (101). Apart from the anti-inflammatory effect, EPS can also stimulate the immune response. EPS derived from yogurt fermented with *Lactobacillus*

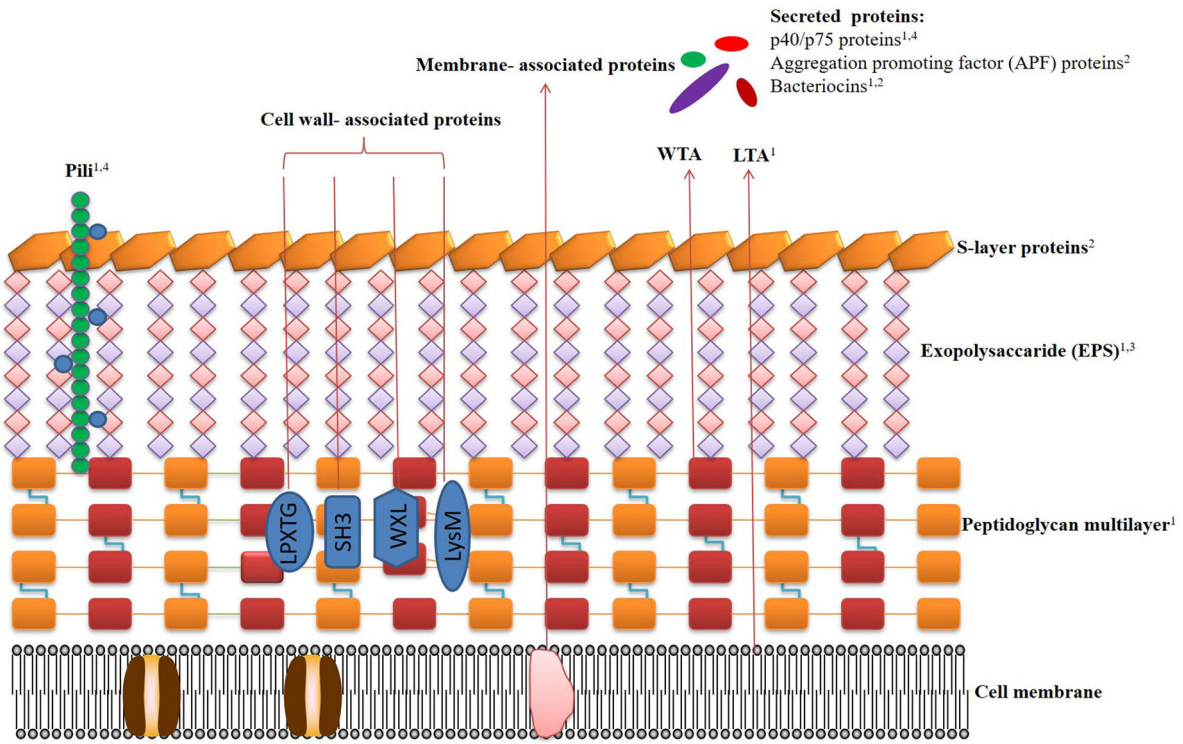

FIGURE 1 | Schematic representation of the cell surface architecture of *Lactobacilli*, the bilipidic cell membrane (CM) with embedded proteins is covered by a multilayered peptidoglycan (PG) shell decorated with lipoteichoic acids (LTA), wall teichoic acids (WTA), pili, proteins, and lipoproteins. Exopolysaccharides (EPS) form a thick covering closely associated with PG and are surrounded by an outer envelope of S-layer proteins. The beneficial effects of the paraprobiotics and postbiotics are denoted by numbers. (1) immunomodulatory effects; (2) antagonistic effects against pathogens; (3) anti-tumor effects; (4) preservation of intestinal barrier. Related references are as follows. **Pili**: immunomodulatory effects (46–48), preservation of intestinal barrier (46, 72). **Protein p40/p75**: immunomodulatory effects (74), preservation of intestinal barrier (17, 75–78). **Aggregation promoting factor (APF) proteins**: antagonistic effects against pathogens (79–83). **Bacteriocins**: immunomodulatory effects (9, 84–87), antagonistic effects against pathogens (88–91). **LTA**: immunomodulatory effects (7, 92, 93). **Peptidoglycan**: immunomodulatory effects (39, 94, 95). **S-layers proteins**: antagonistic effects against pathogens (96–98). **Exopolysaccharides (EPS)**: immunomodulatory effects (99–102), anti-tumor effects (75, 103–106).

delbrueckii (*L. delbrueckii*) subssp. *bulgaricus* OLL1073R-1 induced interferon gamma (IFN-γ) production and activated natural killer (NK) cells in mice (102), which contributed to anti-viral infection effect (114). EPS can also regulate the energy metabolism of host. The EPS isolated from *L. rhamnosus GG* inhibited adipogenesis, and deceased the level of triacylglycerols and cholesterol ester in the liver and serum in mice (115).

Besides to the immunoregulatory effect of EPS, studies also described their anti-tumor abilities. *In vitro* anti-tumor assay of the EPS from *L. plantarum* YW32 proved their powerful inhibitory activity against colon cancer HT-29 cells (63). EPS isolated from *Lactobacillus acidophilus* 20079 strain can regulate both apoptotic and nuclear factor kappa B (NF-κB) inflammatory pathways in human colon cancer and have a potentiality to up-regulate the expression of IKbα, P53 and TGF genes (103). EPSs extracted from *L. casei* M5, *L. casei* SB27, *L. casei* X12, and *L. casei* K11 strains suppressed HT-29 cell growth via induction of G0/G1 cell cycle arrest and apoptosis (104). EPS from *L. plantarum* NCU116 induced c-Jun dependent Fas/Fasl-mediated apoptosis via TLR2 in mouse CT26 cells (105). Moreover, EPS from *L. acidophilus* inhibited the expressions of genes involved in tumor angiogenesis and survival of the colon cancer cell lines *in vitro* (106). Similarly, EPS from *L. acidophilus* LA1 demonstrated their anti-tumor activity *in vivo* against Ehrlich ascites carcinoma cells by suppressing the serum levels of malondialdehyde and nitric oxide (116) and EPS of *Lactobacillus gasseri* strains also showed their capability to inhibit cervical cancer cell growth and modulate immune response (117).

Cell Surface Proteins

Surface layer proteins are one of the most important components of the outermost cell envelope structures on *Lactobacilli* cell surface and other probiotic bacteria species. Cell surface proteins are classified as the proteins which are covalently or non-covalently attached to the cell surface. Recent study indicated that many types of surface proteins including LPXTG proteins, S-layer proteins, pili proteins, moonlight proteins are produced by *Lactobacillus* species including *L. plantarum*, *L. rhamnosus*, *Lactobacillus helvetics* (*L. helveticus*), and *L. acidophilus* (118). These proteins play significant positive roles on the host biological processes.

LPXTG PROTEINS

LPXTG protein is one of the proteins covalently attached to the peptidoglycan of bacterial cell wall. These proteins contain a C-terminal LPXTG signal, and are linked to the cell wall by sortase A (SrtA). In *Lactobacilli,* LPXTG proteins are among the best-known covalent anchored surface proteins. LPXTG proteins were found in many *Lactobacillus* species including *L. plantarum* WCFS1, *L. johnsonii* NCC533, *Lactobacillus sakei* (*L. sakei*) 23K and *L. salivarius* UCC118 (119).

LPXTG proteins from different *Lactobacillus* species have been shown to bind to mucus and epithelial cells, and play major roles in bacteria-host interaction (120). About 12 proteins containing LPXTG motifs were identified from *L. plantarum,* which were involved in adhesion activity (120–122). Their major role was adherence to collagen, fibronectin, chitin, or mucus (123). Furthermore, studies with SrtA mutants of *L. casei* BL23 suggested that SrtA-dependent proteins participated in adhesion of this strain to Caco-2 and HT29 cells (69).

S-LAYER PROTEINS

Many *Lactobacilli* strains, including *Lactobacillus cripatus* (*L. crispatus*) ZJ001 and JCM 5810, *L. acidophilus* ATCC 4356, *Lactobacillus buchneri* (*L. buchneri*) CD034, and *Lactobacillus brevis* ATCC 8287, display a surface coating made of a crystalline, glycoprotein subunits also known as the S-layer (121). S-layer proteins are mostly anchored to peptidoglycan by non-covalent bonds (124). S-layer proteins of *Lactobacilli* account about 15% of total cell wall proteins and they differ from counterparts of other bacteria in their smaller size (25–71 kDa) and higher isoelectric point values (9.4–10.4) (125). In some species of *Lactobacillus*, S-layers with distinctive features can be found, such as glycosylated S-layers in *L. buchneri* and *Lactobacillus kefiri* (*L. kefiri*) (96).

Adhesive S-layers proteins of probiotic *Lactobacilli* can inhibit adherence and infection of pathogenic bacteria. S-layer proteins isolated from *Lactobacilli* were shown to bind to host cell proteins and extracellular matrix (44, 126). The S-layer protein from *L. kefiri* CIDCA 8348 improved the response of macrophages to lipopolysaccharide (LPS) (125) and was able to enhance the ovalbumin-specific immune response by triggering maturation of antigen presenting cells through the recognition of glycan moieties in mice (127). *Lactobacillus paracasei* subp. *paracasei*, *L. rhamnosus*, and *L. casei* strains isolated from natural dairy products are able to inhibit *Shigella sonnei* adhesion to HT-29 cells via their S-layer proteins (128). Similarly, the S-layer proteins from *L. helveticus* fb213, *L. acidophilus* fb116 and *L. acidophilus* fb214 contributed to the adhesion of the *Lactobacillus* strains to HT-29 cells and helped to inhibit the adherence and invasion of *E. coli* ATCC 43893 (96). S-layer proteins of *Lactobacillus* have also been demonstrated to competitively bind the intestinal epithelium *in vivo* and inhibit pathogen infection (97, 98).

PILI PROTEINS

Pili are elongated protein structures protruding outside bacterial cells. Initially pili were considered as special features of pathogens (129), until they were found in *L. rhamnosus*. Pili bind to the intestinal mucusa and promote persistence of Lactobacillus strains in GI tract (130, 131). The SpaCBA pili of *L. rhamnosus* GG were a binding factor to human intestinal mucus, collagen, and intestinal epithelial cell (IEC) lines (46), and SpaC was credited as the major adhesion determinant (71, 107).

Studies also suggested other beneficial effects of pili derived from *Lactobacillus* strains. Mutant of *L. rhamnosus* GG devoid of SpaC induced increased mRNA expression of the pro-inflammatory cytokines IL-8 and TNF-α in Caco-2 cells while wild-type *L. rhamnosus* GG or SpaC alone had little impact on cytokine production (46). The immunomodulatory effect of SpaC was also observed in human fetal intestinal epithelial cell line H4 by modulating TLR-related gene expression (47). Comparative analysis of *L. rhamnosus GG* wild-type and isogenic pili mutants have shown immunoregulatory function of pilli by interactions with monocytes and dendritic cells (46, 48). Similar comparison also demonstrated that pilli can promote pathogen exclusion including pilliated *Enterococcus faecium* (132). Furthermore, SpaCBA pilli have been reported to be involved in promotion of cell proliferation in intestinal crypts, and protection against radiological insults (133). The SpaC pilin of *L. rhamnosus* GG (LGG) has been confirmed to induce the generation of reactive oxygen species (ROS) in epithelium and play a role in stimulating ERK phosphorylation and protecting the gut's epithelial barrier (133).

MOONLIGHTING PROTEINS

Moonlighting proteins include various classes of proteins, including translational elongation factors, metabolic enzymes, ribosomal proteins, and molecular chaperones (134–138). They are found in many species of *Lactobacillus* including *L. crispatus, L. plantarum, Lactobacillus reuteri* (*L. reuteri*) and *Lactobacillus jensenii* (*L. jensenii*) (135, 139–141).

Moonlighting proteins can mediate the colonization of the probiotic strains in intestinal tract. *L. acidophilus* used surface GAPDH to colonize the gut (142). *Lactobacillus* species including *L. plantarum*, *Lactobacillus fermentum* (*L. fermentum*), and *L. jensenii* were found to use moonlighting proteins in competitive exclusion and displacement of pathogens (140). Furthermore, moonlighting proteins including GAPDH, enolase and EF-Tu were involved in plasminogen/plasmin binding and activation (143), which might interfere with the exploitation of plasminogen by gastrointestinal pathogens that express plasminogen receptors or activators, such as *Helicobacter pylori* and *Salmonella* sp. (144).

Postbiotics

As postbiotics, different secretory components of probiotic *Lactobacillus* strains have been reported to mediate beneficial effects, including proteins, peptides, organic acids, and other small molecules. These components can be secreted by live bacteria or released into the host environment after bacteria lysis and confer various physiological benefits to the host.

Secreted Proteins and Peptides

Protein p40 and p75

Protein p40 and p75 were identified from many *Lactobacilli* species including *L. casei, L. paracasei,* and *L. rhamnosus* (145). They are secreted cell wall muramidases and have approximately molecular sizes of 40 and 75 kDa, respectively (74). The positive contribution of these proteins secreted from *Lactobacillus* species has been described in several studies. The protein p40 from *L. rhamnosus* GG showed an immunomodulatry action in mice (74). The p40 transactivated the epidermal growth factor receptor (EGFR) in intestinal epithelial cells, inhibited apoptosis and preserved barrier function in the colon, thereby ameliorating intestinal injury and inflammation (17, 75, 76, 78, 109). Besides, p75 purified from *L. rhamnosus* GG and *L. casei* BL23 have anti-apoptotic activity by inducing the EGF/Akt pathway (145). Furthermore, the p40 and p75 proteins were able to protect the intestinal epithelial tight junctions and barrier functions by a protein kinase (PKC) and MAP kinase-dependent mechanism (76).

Aggregation-promoting factor (APF)

Lactobacillus species have been reported to secrete a number of aggregation promoting factor (APF) proteins, which are extracellular proteins responsible for bridging of conjugal pairs, self-aggregation, maintenance of cellular shape, and co-aggregation with other commensal or pathogenic bacteria (73, 82).

The function of APF from *Lactobacilli* mainly involves host colonization and pathogen exclusion. Previous studies demonstrated that *L. gasseri* SBT2055 decreased adhesion and invasion of *Campylobacter jejuni* (*C. jejuni*) *in vitro* and hindered its infection in chickens via co-aggregation with the pathogens, and the co-aggregation was mediated by proteinaceous cell-surface components (79). Similarly, Yungareva and Urshev (81) also confirmed that APF in *Lactobacillus delbr* (*L. delbr*). subspp *Bulgaricus* had co-aggregation property which inhibited the growth of pathogenic bacteria. APF-2 from *L. gasseri* ATCC 9857 strain contributed to inhibition of the adhesion of *Trichomonas vaginalis* to human vaginal ectocervical cells (80, 81). Furthermore, the presence of high concentration of intracellular GGDEF protein (DgcA) in *L. acidophilus* and a serine/threonine-rich APF protein from *L. plantarum* NCIMB 8826 resulted in increased production of EPS and enhanced the co-aggregation ability (82, 83). The aggregation phenotype enables *Lactobacilli* strains to colonize the GI tract, and to inhibit adhesion of pathogens by competitive exclusion or by co-aggregation with pathogens (62, 146).

Bacteriocins

Bacteriocins are a class of powerful small ribosomally synthesized antimicrobial peptides with bactericidal or bacteriostatic functions (147). Various types of bacteriocins were produced by *Lactobacilli* species, such as lactacin B from *L. acidophilus* and *L. johnsonii*, lactocin from *L. casei,* Lactocin 705 from *L. casei,* Lactoccin G from *L. lactis* and plantaricin from *L. plantarum* (148, 149).

Bacteriocins of probiotic *Lactobacilli* can mediate inhibitory effect against pathogens. Bacteriocin PJ4 produced by *L. helveticus* PJ4 isolated from rat gut microflora was active against enteric pathogen (88) and bacteriocin DT24 produced by vaginal *L. brevis* DT24 was antagonistic against uropathogenic *E. coli* (89). Pangsomboon et al. (150) reported that bacteriocins from *L. paracasei* were able to kill *P. gingivalis*. Moreover, the bacteriocin extracted from probiotic *L. acidophilus* KS40 was able to inhibit urogenital pathogens such as *Gardnerella vaginalis, Streptococcus agalactiae,* and *Pseudomonas aeruginosa* (90). Reuterin produced by *L. reuteri* (6) exerted antimicrobial effects by modifying thiol groups and inducing oxidative stress in bacterial cells (151). *L. salivarius* UCC118, a probiotic strain of human origin, produced bacteriocin Abp118, which mediated the inhibitory effect of the probiotic against *Listeria monocytogenes* infection in mice (91). Additionally, purified bacteriocins from different *Lactobacillus* species have shown anti-infective functions in mice models, demonstrating that bacteriocins can be a promising alternative against gastrointestinal infections (152).

Besides the antimicrobial effects, bacteriocins produced by *Lactobacillus* may also affect host immunity. Plantaricin was identified as the factor in *L. plantarum* WCFS1 that modulate the immune response of DCs (84). Notably, plantaricin can be produced during *L. plantarum* WCFS1 colonization in mice, thus supporting the function of this bacteriocin under *in vivo* conditions (85). Phagocytosis activities of macrophage were improved by bacteriocins isolated from *L. acidophilus* (87). Moreover, bacteriocins can affect the immune function of the host by selectively competing with specific bacterial strains and shaping the microbiota composition (9, 86).

Small Molecules

Small molecules differ from the above mentioned paraprobiotics and protein/peptide postbiotics in that they do not have strain-specific differences in the biochemical characteristics and therefore are generally not responsible for strain-specificity of probiotic functionality. Moreover, different from protein/protein postbiotics, they can be produced by strategies independent of the probiotic strains. However, subsets of the probiotic effects are mediated by small molecules. Therefore, in this review, we also categorized small molecules as postbiotics, and summarized their beneficial effects.

Short chain fatty acids

SCFAs are produced by gut microbiota from indigestible food components such as fiber, oligosaccharides and polysaccharides via different metabolism channels (153, 154). The SCFAs have a wide range of positive effects on the host, such as providing energy sources for colonic epithelium cells (155), maintaining metabolic homeostasis (156), regulating T regulatory cells (157, 158), and anti-inflammatory effects (159–162). Generally they are essential for the health and well-being of the host when present in sufficient amounts (163). Studies showed that *Lactobacillus* strains can produce different types of SCFAs. *L. rhamnosus* GG and *L. gasseri* PA 16/8 produce propionate (163, 164).

Moreover, SCFAs have been associated with the beneficial effects of probiotic *Lactobacillus* strains in some research. Dhaliwal et al. (165) confirmed that supplementation of mice with *L. plantarum* showed an increase in acetate and butyrate levels and reduced intestinal permeability and monoamine oxidases in the brain. SCFAs-promoting probiotic *L. johnsonii* L531 treatment have been shown to control *Salmonella* infection and maintaining metabolic homeostasis in pig (166). In a screening of LAB to reduce cholesterol levels, the strain of *L. plantarum* CECT 7529, which produced higher quantities of propionic and butyric acids, showed excellent properties for reducing cholesterol levels (167). Furthermore, probiotic strains *L. salivarius* FP25 and FP35, and *L. reuteri* NCIMB exhibited inhibitory effect on colon cancer cell proliferation, which was mediated by the production of SCFAs (168, 169).

Conjugated linoleic acid (CLA)

Studies showed that many *Lactobacillus* species are able to synthesize conjugated linoleic acids (CLAs) (170, 171). The ability of *L. rhamnosus* PL60 to produce *cis*-9, *tra*-11 and *tra*-10, *cis*-12-CLA in humans was the first report indicating that probiotic bacteria produce CLA (172). Further studies showed that some Lactobacilli species isolated from GI tract of human and animals, including *L. rhamnosus*, *L. acidophilus* and *L. plantarum*, are CLA producers (173, 174).

CLA inhibited the growth of HT-29 and Caco-2 cancer cell lines *in vitro* (175). Proliferation of MDAMB-231 cells was inhibited by *L. plantarum*-produced CLA in a dose dependent manner (176). *In vivo* administration of CLA to rats could decrease the occurrence of colonic tumors and increase the apoptotic indices (177). Moreover, CLA has been shown to reduce the incidence of colonic, skin, mammary, and prostate carcinogenesis in animal models (178).

CLAs produced by probiotic *Lactobacillus* have remarkable anti-tumor effect. CLA inhibited the growth of HT-29 and Caco-2 cancer cell lines *in vitro* (175). Proliferation of MDAMB-231 cells was inhibited by *L. plantarum*-produced CLA in a dose dependent manner (176). *In vivo* administration of CLA to rats could decrease the occurrence of colonic tumors and increase the apoptotic indices (177). Moreover, CLA has been shown to reduce the incidence of colonic, skin, mammary, and prostate carcinogenesis in animal models (178).

Neurotransmitters

Gut bacteria contribute to the proper function of gut-brain axis by producing neurotransmitters, such as γ-aminobutyric acid (GABA), glutamate, serotonin (5-HT), dopamine (DA), norepinephrine, histamine and acetylcholine (179). Particularly, *Lactobacillus* can produce multiple neurotransmitters, such as GABA (180–186), serotonin (181), catecholamines (181), dopamine (181), and acetylcholine (187). Different probiotic *Lactobacillus* strains have been reported to confer beneficial effects on mental health, acting as "psychobiotics," including *L. paracasei* (188), *L. helveticus* (189, 190), *L. plantarum* (165, 191), and *L. rhamnosus* (192). Moreover, studies have shown that histamine and dopamine produced by gut commensal *Lactobacillus* imparted significant role in sleep related disorders and regulates neuronal signaling in depression, anxiety related conditions disease (193), suggesting that the beneficial effects of probiotic *Lactobacillus* on mental health might be attributable to the neurotransmitters production.

INTERACTION OF PARAPROBIOTICS AND POSTBIOTICS WITH THEIR RECEPTORS ON HOST CELLS

The beneficial effects of paraprobiotics or postbiotics are mediated through an interaction between the microbial products and host. Probiotic *Lactobacilli* possess conserved MAMPs, including peptidoglycan, LTA, S-layer protein A (SlpA), EPS, and genomic DNA, which can be recognized by pattern recognition receptors (PRRs), induce downstream signaling cascades that confer the beneficial functions (5).

The importance of Toll-like receptors (TLRs) and Nucleotide-binding oligomerization domain-like receptors (NLRs) in mediating differential host interaction with paraprobiotics and probiotics has been widely acknowledged (107, 194). In this review we summarize four types of the PRRs that play principal roles in the regulation of the host's immune response and these different types of PRRs can bind to specific paraprobiotics or postbiotics of *Lactobacillus* strains (**Figure 2**).

Toll-Like Receptors (TLRs)

TLRs recognize distinct families of MAMPs. For instance, TLR2 recognizes LTA and peptidoglycan; TLR2/TLR4 recognize bacterial EPS with the help of RP105/DM1; TLR9 is responsive to unmethylated CpG oligonucleotide (CpG-ODN) (195) (**Table 2**). *L. reuteri* DSM 17938 strain showed a positive effect against necrotizing enterocolitis via TLR2 (203). TLR2 recognized the LTA of *L. plantarum*, and attenuated Pam2CSK4-induced IL-8 expression (46).

The EPS of *L. delbrueckii* TUA4408L can act as TLR2 and TLR4 ligands, and exert anti-inflammatory activities in porcine IECs by modulating MAPK and NF-κB signaling pathways (197). *L. plantarum* N14 EPS reduced inflammation in intestinal epithelial cells depending on RP105/MD1 complex (a member of TLR family). (101). Similarly, *L. rhamnosus* GG and its components (surface layer protein and EPS) inhibited MAPK and NFκB signaling and alleviated LPS-induced inflammatory cytokines in porcine intestinal epithelial cells by modulating TLR expressions (204).

Nucleotide-Binding Oligomerization Domain-Like Receptors (NLRs)

NLRs constitute a large family of PRRs and includes a number of subfamilies, which can be distinguished depending on the N-terminal effector domains (195). Two well-studied NLR proteins are NOD1 and NOD2. The NOD1 recognizes molecules containing D-Glu-mDAP (205), whereas NOD2 are vital for the regulation of NAM-D-Ala-D-Glu unit of the molecules (206). Recognition of muropeptide from *Lactobacilli* by NOD2 can induce anti-inflammatory properties and protect mice from colitis development (94). Different types of signaling

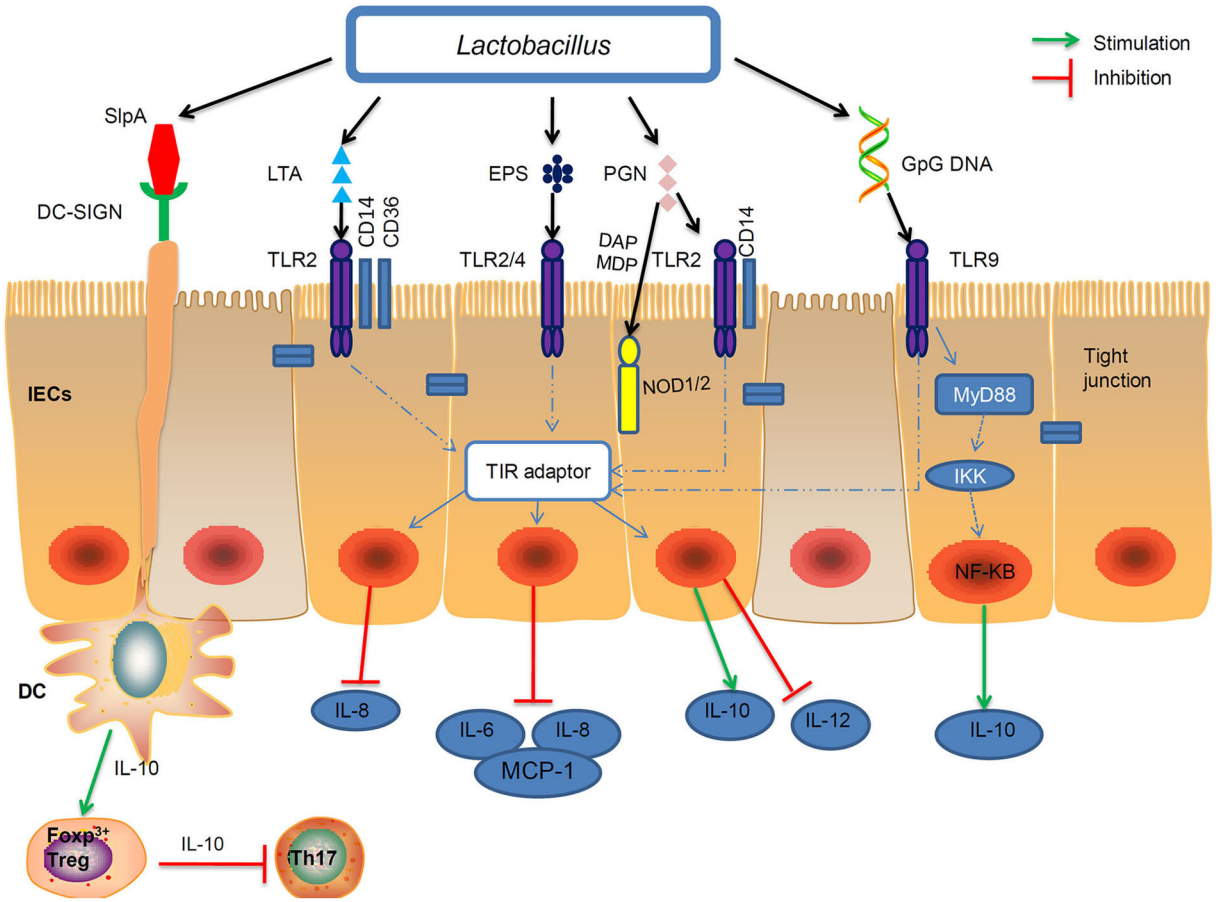

FIGURE 2 | Interactions of the MAMP of *Lactobacillus* with PRRs of the epithelial and immune cells of the host. Probiotic *Lactobacillus* possess conserved microbe-associated molecular patterns (MAMPs), including peptidoglycan, lipoteichoic acids (LTA), S-layer protein A (SlpA), exopolysaccharides (EPS), and genomic DNA which can be recognized by certain pattern recognition receptors (PRRs). Peptidoglycan and LTA interact with TLR2. Moreover, specific components of peptidoglycan, such as meso-DAP and MDP, are recognized by NOD1 and NOD2, respectively. The EPSs of *L. delbrueckii* TUA4408L, act as TLR2 and TLR4 ligands to exert anti-inflammatory activities by inhibiting the production of IL-6, IL-8, and MCP-1. On the apical side of IECs, CpG-DNA stimulated TLR9 interacts with MYD88 and the inhibitor of NF-κB kinase (IKK) complexes, which may induced IL-10 expression. Binding of SlpA to the DC-SIGN (dendritic cell-specificICAM3-grabbing non integrin) receptor can induce IL-10 production in DCs and development of T cells. IEC, intestinal epithelial cell; DC, dendritic cell; Treg, T regulatory cell; Th, T helper cell; MCP-1, monocyte chemoattranctant protein-1.

molecules from *Lactobacilli* species including the fragments of peptidoglycan were sensed by NODs (207), and this sensing results in the activation of NF-κB and antimicrobial activity (208).

C-Type Lectin-Like Receptors (CTLRs)

CTLRs recognize carbohydrates molecules, through one or more carbohydrate recognition domains (CRDs) (209). The sugar moieties found in the glycan backbone of the bacterial peptidoglycan bind CTLRs (210). After the ligand recognition, specialized CTLRs trigger or inhibit wide ranges of signaling pathways, thus modulate diverse immune responses (211).

DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN) is a CLR expressed mainly on dendritic cells (DCs) and recognizes mannose- and fucose-containing glycans that are present on many species of *Lactobacilli* bacterial cell surfaces. DC-SIGN was previously shown to bind *L. acidophilus* SlpA *in vitro* (200). SlpA-DC-SIGN interaction induced IL-10 production in DCs promoted of T cells that secrete high amounts of IL-4, thereby decreasing the Th1/Th2 ratio (200). Further, *in vivo* role of the SlpA-induced protective immune regulation was demonstrated (212).

G-Protein-Coupled Receptors (GPCRs)

The best characterized GPCRs are GPR41 and GPR43, which are highly expressed by epithelial cells, adipocytes, enteroendocrine cells and the cells of the sympathetic nervous system (213), and are mainly activated by SCFAs (214). Butyrate and propionate produced by microbiota in the gut acted with GPR43 and regulated the accumulation of Foxp3^{+} Treg cells (215). The recognition of SCFAs by GPR109A has also been reported. For instance, activation of the GPR109A receptor by butyrate induced the differentiation of regulatory and IL-10-producing T cells, which suppressed colonic inflammation and carcinogenesis by promoting anti-inflammatory properties in colonic macrophages and dendritic cells (216). Furthermore, SCFAs produced by gut

TABLE 2 | Receptors, ligands, and immunological effects.

Receptors	Ligands	Probiotic effects	Model	References
TOLL-LIKE RECEPTORS (TLRs)				
TLR2	Peptidoglycan	Down-regulate IL-12	Mouse cell lines	(94)
TLR2	LTA	Down-regulate IL-8, balance IL-10/IL-12	Human epithelial Caco-2 cell line	(92, 196)
TLR2, TLR4, RP105/MD	EPS	Down regulate IL-6, IL-8, MCP-1,	Porcine intestinal cell lines	(101, 197)
TLR9	Unmethylated CpG DNA	Suppress NF-κB signaling pathway	Porcine cell lines, Mouse cell lines	(198, 199)
NUCLEOTIDE-BINDING OLIGOMERIZATION DOMAIN-LIKE RECEPTORS (NLRs)				
NOD1 and NOD2	Meso-DAP, MDP	Up-regulate IL-10, suppress the production of IL-12	Mouse cell lines	(94)
C-TYPE-LECTIN RECEPTORS (CLRs)				
DC-SIGN	SlpA	Up-regulate IL-10, IL-4	Human cell lines	(200)
G-PROTEIN-COUPLED RECEPTORS (GPCRs)				
GPR41 GPR43 GPR109A	Acetate, propionate, and butyrate	Down-regulate TNF-α, IL-6, IL-12, and NO up-regulate IL-10	Human cell lines	(201); (202)

microbiota may regulate lipid metabolism, glucose homeostasis and insulin sensitivity through GPCR signaling (156).

CONCLUSIONS

Paraprobiotics and postbiotics derived from *Lactobacillus* species consist of a wide range of effector molecules. These products and byproducts of probiotic *Lactobacillus* have been found to possess magnificent beneficial functions including preservation of epithelial barrier, anti-tumor effect, immunomodulation, and antagonistic effects against pathogens. Furthermore, they have various advantages compared with probiotics, including clear chemical structures and safety dose parameters, as well as longer shelf life (217, 218). Therefore, the use of paraprobiotics and postbiotics may represent a valid and safer alternative to live probiotic bacteria, and have exhibited good potential to replace probiotics (219, 220).

The mechanisms underlying the beneficial effects have been less known, especially the signaling pathways downstream the interaction of paraprobiotics/postbiotics and PRRs, which deserve more investigation. Furthermore, the structure-activity relationship (SAR) of paraprobiotics and postbiotics will be an interesting topic, which may guide the functional improvement of these probiotic components, by either chemical or biological strategies.

Currently the application of postbiotics and paraprobiotics in human food, animal feed and pharmaceutical industries is increasing and several paraprobiotic and postbiotics products derived from *Lactobacill* species are commercially available for prevention or treatment of some diseases (221–225). Nevertheless, more evidence is needed to validate the beneficial effects of paraprobiotics and postbiotics. Current advancement of molecular technologies such as multi-omics have been promoting the identification of more paraprobiotics and postbiotics from probiotic *Lactobacillus* strains. Moreover, novel probiotics from other family or phylum are being discovered and studied, such as commensal bacterium isolated from the intestine of both human and animals (226, 227). The techniques and experience of paraprobiotics and postbiotics discovery from probiotic *Lactobacilli* may guide the investigation of novel functional components derived from the new probiotics. Collectively, paraprobiotics and postbiotics have good potential as prophylatctic or therapeutic agents as well as functional food or feed additives for human or animal use.

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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Short-Chain Fatty Acids: A Soldier Fighting Against Inflammation and Protecting From Tumorigenesis in People With Diabetes

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Converging evidences showed that people with diabetes mellitus (DM) have significantly higher risk for different cancers, of which the exact mechanism underlying the association has not been fully realized. Short-chain fatty acids (SCFAs), the fermentation products of the intestinal microbiota, are an essential source for energy supply in gut epithelial cells. They have been reported to improve intestinal barrier integrity, prevent microbial translocation, and further dampen inflammation. Gut dysbiosis and reduction in SCFA-producing bacteria as well as SCFAs production in the intestine are commonly seen in metabolic disorders including DM and obesity. Moreover, inflammation can contribute to tumor initiation and progression through multiple pathways, such as enhancing DNA damage, accumulating mutations in tumor suppressor genes Tp53, and activating nuclear factor-kappa B (NF-κB) signaling pathways. Based on these facts, we hypothesize that lower levels of microbial SCFAs resulted from gut dysbiosis in diabetic individuals, enhance microbial translocation, and increase the inflammatory responses, inducing tumorigenesis ulteriorly. To this end, we will discuss protective properties of microbial SCFAs and explore the pivotal roles SCFAs played in the link of DM with cancer, so as to take early precautions to reduce the risk of cancer in patients with DM.

Keywords: short-chain fatty acids, gut dysbiosis, inflammation, diabetes, cancer

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INTRODUCTION

Diabetes mellitus (DM) is characterized by different metabolic abnormalities, including hyperglycemia, insulin secretion deficiency, insulin dysfunction, and energy metabolism disturbances (1, 2). The World Health Organization (WHO) reported about 422 million people have DM in the world. Morbidity and prevalence of DM have been steadily rising over the past few decades. Aside from DM, cancer ranks as the second main cause of death in the developed world and the third cause for death in developing nations (3). DM and cancer share several common characteristics, such as microbial imbalance and bacterial translocation (4–6). Unfortunately, these two diseases are frequently coexistent and can affect each other, worsening the prognosis of patients.

Generally compared with non-diabetic people, the incidence of cancer in diabetic patients increased by approximately 20–25%, depending on specific cancer type and site (7, 8), which has been observed in both Asian and Western populations (9). People with diabetes accompanied by cancer also experienced higher mortality risk, in contrast with the group with only cancer (10). Practically speaking, when comparing to the general population, the significantly increased risk of cancer in diabetic individuals has been proved in hepatocellular and pancreatic carcinomas, which were 2.5 and 1.94 times higher respectively (11, 12). Friberg et al. demonstrated that in diabetic women, the risk for endometrial cancer nearly doubled (13). The epidemiological literature indicates that patients with DM are at modestly increased risk (by 19–42%) of developing colorectal, gastric, kidney, breast, and bladder cancers (14–16). Numerous studies have also declared the positive associations of DM with non-Hodgkin lymphoma, leukemia, and myeloma, which is about 19–22% higher in patients with only type II DM (17–19). Regarding type I DM cohorts, in comparison with the non-diabetics, increased risk of several cancers might be reported in multiple research cohorts, yet inconsistent if all studies are considered (20–22).

However, regarding the existing correlation of DM with cancer, the mechanisms remain to be established. Identifying and utilizing the intermediate links between the two diseases are essential to reduce the risk of cancer and improve the symptoms of DM. Short-chain fatty acids (SCFAs), as functional microbial metabolites (23), might be a possibility to unlock the tie. Herein, we will go further behind this correlation and focus on SCFAs, which may be involved in the progress of DM developing into cancer.

HYPOTHESIS

Based on recent evidences, we hypothesize that lower abundance of SCFAs resulted from gut microbial dysbiosis in diabetics could induce the destruction of the gut mucosal barrier, increase microbial translocation, and promote inflammation responses, further boosting inflammatory-malignant transformation and enhancing risk of tumorigenesis (**Figure 1**).

GUT MICROBIOTA DYSBIOSIS AND LOWER SCFAS IN DM

The intestinal barrier is the functional defense line, consisting of microbiota, intestinal epithelial cells (IECs), and mucosal immunity (24). The gut microbiota are collectively referred to as eukaryotes, archaea, and bacteria, among which bacterial phyla are the most abundant (25). Investigations identified that keeping the diversity and quantity of human gut microbiota, which can protect the host against pathogens by competition for niches and nutritive supply, improvement of immune functions, and regulation of metabolism, is of critical importance in maintaining intestinal homeostasis (26, 27). *Akkermansia muciniphila* was reported to improve intestinal epithelial monolayer integrity by stimulating colonic mucin secretion, increasing the thickness of the gut mucus layer, and binding directly to intestinal cells (28, 29). On the contrary, dysregulation of gut microbiota composition, also called dysbiosis, could impair the balance between the commensal species and various pathogens, as well as decrease the release of metabolic SCFAs and antimicrobial molecules such as bacteriocins (30). Depleted Firmicutes and increased Proteobacteria such as *Enterobacter cloacae* and *Enterobacter* species contribute to the disruption of IECs, which in turn induces the translocation of intestinal microbiota and their toxins, leading to infectious threats (31, 32). Dysbiosis may also regulate IECs to release intestinal miRNAs, which could orchestrate the immune responses *via* TLR dependent pathways or PRR families to fight against pathogens (33). Other than SCFAs and bacterial toxins, microbiota might influence the intestinal barrier through other signal pathways, including bile acids metabolism and endocannabinoid system (34, 35).

Emerging evidences indicated DM is significantly correlated with gut microbiota dysbiosis, which harms the integrity of the gut wall and promotes the shift of endotoxemia from the intestinal cavity into the circulatory system, hence triggering inflammation, autoimmune responses, and oxidative stress (36). Larsen et al. demonstrated that microbial dysbiosis could provoke changes in composition and distribution of gut microorganisms, particularly intestinal bacterial species in the mucosa, and metabolic activities (37). Compared to non-diabetics, *Lactobacillus spp* and Betaproteobacteria groups were highly enriched in diabetics, which was positively connected to plasma glucose and could trigger inflammation effect in DM (38–40). Considerable studies indicated that the abundance of *Akkermansia muciniphila*, Firmicutes, and *Clostridium* spp was significantly decreased among individuals with DM, inducing unfavorable effects on nutrient metabolic control, glucose tolerance, and inflammation responses (41–43). In addition, the diabetics possessed lower abundance of SCFA-producing organisms, including *Roseburia intestinalis* and *Faecalibacterium prausnitzii*, which led to reduced anti-inflammatory SCFAs levels, especially butyrate (37, 44, 45). Similar results have been observed in animal studies describing that the levels of SCFA-producing bacteria and SCFA production declined remarkably in diabetic mice versus the diabetes-free group (46, 47). Deficiency in SCFA production could promote intestine microbes to spread into the systemic circulation, thereby inducing or aggravating systemic inflammatory responses (48, 49).

Notably, intestinal SCFA producers and their metabolites SCFAs are reported to play crucial roles in the pathophysiology of DM. Compared to healthy controls, type 1 diabetes mellitus (T1DM) patients harbored decreased population of SCFA-producing bacteria and circulating SCFAs, in line with markedly decreased genes contributing to SCFAs synthesis, which is associated with disturbed microbiota composition (50–52). Gut metagenome data also revealed that subjects with type 2 diabetes mellitus (T2DM) were characterized by a decrease in

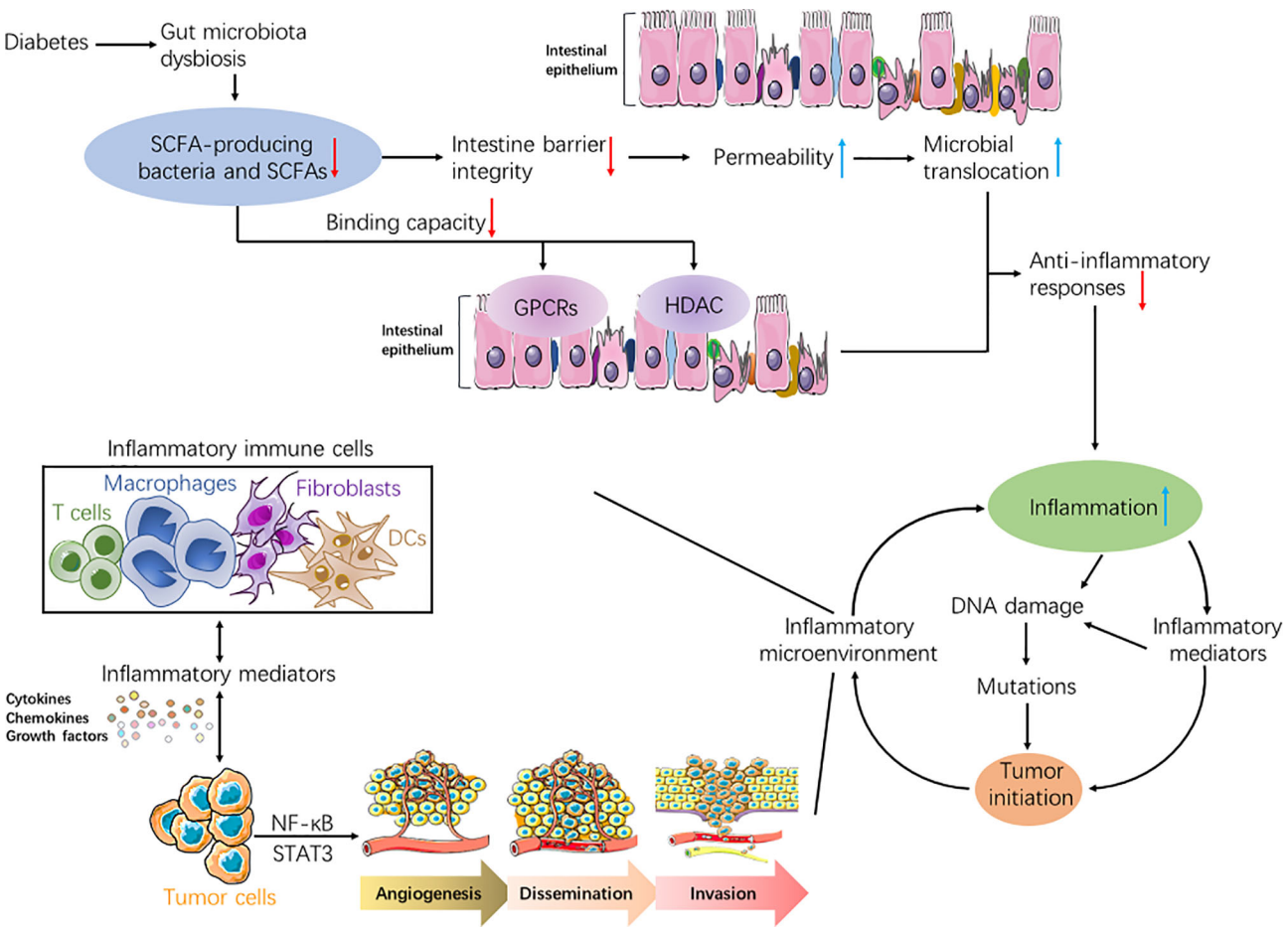

FIGURE 1 | Flowcharts representing the association of diabetes, short-chain fatty acids (SCFAs), inflammation, and tumor. Due to gut microbiota dysbiosis in people with diabetes, both SCFAs-producing bacteria and the produced SCFAs decreased. Reduction in SCFAs could weaken anti-inflammatory responses in diabetics through diverse mechanisms, such as increasing gut permeability, inducing microbial translocation, and attenuating binding capabilities of SCFAs to both GPCRs and HDAC. Hence, the inflammation was exacerbated in diabetic patients. Increased inflammation may lead to tumorigenesis in normal cells through accumulated genetic mutations, which proceeded from DNA damage. Moreover, various inflammatory immune cells can secrete abundant inflammatory mediators such as cytokines, chemokines, and growth factors, which may activate key transcription factors, including NF-κB and STAT3. This could initiate tumor growth in normal cells, or boost malignant processes in tumor cells including angiogenesis, dissemination, and invasion. In turn, inflammatory reactions were aggravated under sustained tumor-associated inflammation, thus forming a vicious and positive feedback loop between inflammation and tumor.

SCFA-producing microbiota and SCFA abundance, issued from altered gut microbiota, impaired intestinal barrier, and increased plasma levels of lipopolysaccharides (LPS) (53–55). For diabetics, the favorable effects of SCFAs were mainly identified in reducing serum glucose levels, improving insulin resistance, mitigating inflammation, and enhancing secretion of protective glucagon-like peptide 1 (GLP-1) (56–58). Conversely, dietary interventions with SCFAs were shown to alleviate T1DM in mice models, as evidenced by decreased auto-immune T cell counts, suppressed B cell proliferation, and expanded autoimmune FoxP3+ Treg cell repertoire in the colon, spleen, and systemic lymph nodes (46, 58). By providing SCFA-releasing diets to T2DM patients, it could significantly improve metabolic disorders by enriching SCFA-producing microbes mass, ameliorating individual glucose tolerance, and reducing levels of hemoglobin A1c (59–61). Herein, we discuss the latest developments in the protective effects of SCFAs and appreciate their potential involvement in diseases.

EFFECTS OF SCFAS ON INFLAMMATION, IMMUNITY, AND METABOLISM

SCFAs, predominantly acetate, butyrate, and propionate, are anaerobic fermentation metabolites of fiber produced by intestinal microorganisms (62). SCFAs act as the principal energy source for colorectal cells, enhancing intestine epithelium integrity (63). Besides, as leading messenger molecules between gut microbiota and host health, SCFAs can enter the blood circulation, protect the gut barrier, regulate

inflammatory responses, and influence immune functions of distal tissues (64).

SCFAs can promote the intestinal epithelium function, mainly by hypoxia-inducible factor-1 (HIF-1), which is a transcription factor stabilizing intestinal epithelial barrier (65). Antibiotic-mediated microbiota depletion reduces intestinal levels of HIF and causes epithelium impairment, which could be restored by supplementing butyrate. However, the effects of butyrate on gut epithelial barrier protection vanished when lacking HIF (29). In addition, SCFAs can restore the intestinal barrier integrity by inducing gene expressions of inter-epithelial junction protein Claudin-1, and activating other transcription factors such as STAT3 and SP1, even in inflammatory conditions (66, 67). Another involved mechanism is that SCFAs could induce IECs to produce antimicrobial peptides (AMPs), recruit neutrophils and anti-inflammatory cytokines, suppress activation of NLRP3 inflammasome, and further protect the intestinal barrier from damage by pathogens (68, 69).

When SCFAs were supplemented *in vivo*, they were shown to preserve the molecular barrier of intestinal epithelium. Vieira et al. reported that oral SCFAs could significantly improve intestinal trophism and inhibit leukocytes and other immunocytes infiltration, further attenuating the inflammatory state of gut barrier in rats with acute ulcerative colitis (70). SCFAs treatment may also constitute substrates for gut mucosa and ameliorate intestinal damage, by restoration of glutathione levels and reduction in proinflammatory mediators, including nitric oxide (NO) and tumor necrosis factor-α (TNF-α) in rat models of colitis (71). In addition, rectal administration of SCFAs could promote mucus secretion and intestinal repair, by increasing abundance of mucus-associated bacteria and expression of genes required for pathogens elimination, such as IL-17A and IL-1β in mice with enteritis (72, 73).

Säemann et al. reported that administration of SCFAs could not only reduce secretion of pro-inflammatory markers such as TNF-α, IL-6, and IL-12 but also increase release of anti-inflammatory markers including IL-10 in cultured human peripheral blood mononuclear cell (PBMCs) *in vitro* (74), providing important clues to cognize the anti-inflammatory actions of SCFAs. With evidence-based potentiated generation of anti-inflammatory regulatory T cells, inhibited release of reactive oxygen species, and suppressed production of pro-inflammatory cytokines including TNF-α and IL-1β, anti-inflammatory effects of dietary SCFAs have been confirmed in different animal models, such as steatosis, allergic airway inflammation, and chronic kidney disease (CKD) (64, 75, 76). In addition, clinical investigations revealed that the inflammatory potential, analyzed by the TNF-α/IL-10 ratio, was significantly higher in the diabetic group than the healthy ones, which may be counteracted by dietary fiber that can produce SCFAs (77, 78), corroborating the inflammation inhibitory potential of SCFAs. In most cases, reduced inflammatory conditions and improved immunity are inseparable.

Several mechanisms have already been explored on the anti-inflammation influences of SCFAs (1). Permeability: Increased permeability is believed to induce microbial translocation, which could trigger an inflammatory cascade (79). Chen et al. confirmed that the representative SCFA, butyrate, is a crucial substrate for promoting epithelial cell growth, which can maintain the colonic epithelium, prevent excessive gut permeability, and even induce innate immune responses to injury and invasive microorganisms if necessary (80) (2). Nuclear factor-kappa B (NF-κB): NF-κB is a critical transcription factor for inducing expressions of multiple inflammation related genes. Studies have shown that butyrate can inhibit NF-κB activation in human macrophages and epithelial cells (81, 82) (3). Histone deacetylation (HDAC): HDAC inhibitors were initially developed as cancer-combating agents. Nowadays HDACs inhibitors are attracting much more interest as anti-inflammatory agents, independent of their known proapoptotic or cell cycle arrest actions on malignant cells (83). SCFAs are natural HDACs inhibitors, facilitating expressions of anti-inflammatory genes in the immune cell, promoting T lymphocytes as they differentiate into effector T cells such as Th1 and Th17 cells subsets, and boosting their immune responses in inflammation (84, 85). Kim et al. also revealed that by promoting activation and differentiation of B cells to plasma B cells, SCFAs accelerated the production of most antibodies types, including IgG and IgA (86) (4). G-protein coupled receptors (GPCRs): SCFAs could activate GPR41 and GPR43 in intestinal epithelial cells, leading to transmission of mitogen-activated protein kinase signaling, and rapid secretion of chemokines and cytokines (87, 88). Singh et al. showed that *via* activating GPR109A in macrophages and dendritic cells, SCFAs make them highly efficient inducers of regulatory T cells, particularly $FoxP3^+$ T cells, to limit inflammation and control carcinogenesis (88). So SCFAs not only act as anti-inflammatory bacterial metabolites but also function as immune boosters to prepare the host to better exterminate pathogens.

Furthermore, SCFAs are among the most extensively studied microbial metabolites that intervene in host metabolism. By binding to GPR43 and GPR41, SCFAs are able to raise plasma levels of GLP-1, peptide YY (PYY), and leptin, resulting in reduced food intake, enhanced glucose metabolism, and improved glucose homeostasis (89–91). Fascinating animals researches also indicated that butyrate and propionate may both activate gene expression germane to intestinal gluconeogenesis, through cAMP-dependent pathway and GPR41-dependent gut-brain circuits respectively, eventually improving glycaemia control and ameliorating insulin sensitivity (92, 93). Another vital function of SCFAs is intracellular metabolic integration to produce energy such as adenosine triphosphate (ATP) (94). Canfora et al. demonstrated that infusions of SCFA mixtures in the colon boosted energy consumption, enhanced fat oxidation, and decreased lipolysis in metabolic profiles (95).

In order to achieve these benefits, the production of SCFAs can be encouraged in various ways, such as natural sugar and high-fiber intake (96, 97). However, it is necessary to determine the dosage range of SCFAs before they can be applied in clinic, as superfluous SCFAs might exert some adverse effects, such as accelerating cholesterol synthesis and lipid accumulation within the liver (98, 99). Excessive acetate and butyrate may also be involved in host fat storage by enhancing energy intake and intestinal polysaccharide

degradation, thereby contributing to weight gain and obesity phenotype in obese mice (100, 101).

Considering their extreme importance in resisting inflammation in the gut, SCFAs are regarded with mighty potential for certain diseases including metabolic conditions and cancer, providing strong theoretic foundation to launch SCFAs-based clinical trials for cancer treatment. However, the causative role of SCFAs abundance in diabetics with respect to carcinogenesis needs further elucidation.

ROLES OF INFLAMMATION AND SCFAS IN THE CONTEXT OF CANCER

Inflammation usually involves the activation, recruitment, and functioning of innate and adaptive immune cells, which is essential for the host to defend against pathogens, repair damaged tissues, and regulate tissue homeostasis (102). Acute inflammation is protective and normally self-limited, which would be terminated after elimination of harmful triggers or completion of the restorative process (103). This self-limiting property of acute inflammation was also verified by Bannenberg and his colleagues in mouse peritonitis models, induced by zymosan (104). Once acute inflammatory responses are out of control and cause tissue damage, it will amplify inflammation and progress to chronicity (105), hence predisposing people to cancer development. More importantly, inflammatory immune cells together with fibroblasts and vascular endothelial cells constitute the stroma network for cancer cell survival, namely the tumor microenvironment (TME) (106), which can boost tumor occurrence, tumor promotion, malignant transformation, and metastatic transmission (107, 108). In general, tumor extrinsic inflammation can be caused by certain factors including infection, metabolic diseases, autoimmune diseases, and smoking, while tumor intrinsic inflammation can be triggered by cancer-related gene mutations or by recruiting and activating inflammation-fighting cells (109). Inflammation, irrespectively of its inducement or appearance, owns a significant effect on carcinogenesis. Typical pro-inflammatory cytokines IL-17 and IL-23, which can promote tumor development and progression respectively, were both upregulated in mouse models of colorectal cancer (CRC) (110, 111). In turn, tumorigenesis may enhance pro-tumorigenic inflammation, illustrating the essential circle between inflammation and cancer. Collectively, epidemiological studies showed that inflammation is linked to the initiation of around 20% of cancers (112). To be specific, chronic inflammations caused by the hepatitis virus and *Helicobacter pylori* are associated with the majority of hepatocellular and gastric carcinomas respectively (113–115). Approximately 2% of CRC develops in patients with ulcerative colitis (UC) (116, 117). Chronic airway inflammations with tobacco smoke and airborne particulates are the major risk factors for lung cancer (118, 119).

Although the predisposing factors or sources may vary, inflammation always goes together with increased risk of cancer, oncogenic transformation, and malignant progression *via* multiple approaches (1). Enhancement of DNA damage. During inflammatory conditions, both inflammatory and epithelial cells can release chemicals like reactive oxygen and nitrogen species (RONS), which may cause DNA lesions (120). Furthermore, the generated DNA damage signals and cytotoxicity can promote inflammation, inducing a continuous vicious circle between DNA lesions and DNA repair, which can further enhance DNA damage. Subsequently, this feedback loop induces genetic mutations, genome instability, and eventual tumorigenesis (121, 122). Chen et al. reported that 7,12-Dimethylbenz[a]anthracene (DMBA), a mighty genotoxic agent, could activate the cGAS–cGAMP–STING pathway, inducing inflammation-driven cutaneous carcinogenesis in mouse models (123). Singhal et al. have manifested that DMBA was engaged in mammary carcinogenesis as well as the distant metastasis in skin-tumor-sensitive (STS) female mouse strains, mimicking DMBA-induced human breast cancer (124) (2). Inactivation of anti-oncogene and activation of oncogenes. Without additional inducers, chronic inflammation can induce accumulated mutational in-activations of tumor suppressor genes such as Tp53 in the epithelial cells, leading to upregulation of microtubule modulin stathmin1 and increased chemoresistance in breast and colon cancer cell lines or patient specimens (125–127). Moreover, accumulation of activated mutations in epithelial oncogenes (K-ras and c-Myc) can be promoted by inflammation-induced DNA damage or inflammatory cytokines, which can also cooperate with other inflammatory stimuli to manifest cancerogenic activities, such as enhancing mutagenesis, promoting tissue injury, and ultimately carcinogenesis (128, 129) (3). Regulation of signaling pathways. Both NF-κB and signal transducer and activator of transcription 3 (STAT3) have been identified as cardinal inflammatory signaling molecules during carcinogenesis. Survival signals activated by NF-κB and STAT3 were enhanced in mutated epithelial cells, which protected cells against the attack of cytotoxic T lymphocytes, boosted malignant clones, and enabled tumor outgrowth (130–132). Otherwise, programmed cell death protein-1 (PD-1) pathway was activated to deliver inhibitory signals, which may be evoked by inflammatory signals interferon-gamma (IFN-γ), K-ras mutations, or K-ras/Tp53 co-mutations. This could drive T cell exhaustion, generate a tolerant microenvironment, and help cancerous cells escape immune surveillance and survive, particularly for patients with lung adenocarcinoma (133–135) (4). Modulation of inflammatory mediators. Cytokines, chemokines, and growth factors are the predominant cell-signaling molecules produced by multiple cells in the inflammatory microenvironment and are fundamental to tumor development in different stages (136). Dash reported that TNF-α was among the chief cytokines in inflammatory responses, which rendered epithelial to mesenchymal transition (EMT), accelerated cancerous cells invading process, and elicited other inflammatory proteases to orchestrate inflammatory conditions (137). Tumorigenic cytokines such as IL-6 and IL-11 could directly act on tumor cells, enhancing cell proliferation, stimulating angiogenesis, and expanding cancer stem cell (CSC) population, in mouse xenograft models of gastrointestinal and breast cancers (138, 139). Calon et al. identified that IL-11 was also involved in summoning myeloid cells such as fibroblasts and tumor growth factor-β (TGF-β), which could facilitate cancer cell migration, promote tumor invasion, and assist metastatic transition in colorectal carcinoma of mice (140). Regarding the similarity of inflammatory

processes in distinct cancers, preventing or controlling inflammation may be an imperative treatment for cancer.

Numerous studies have demonstrated that some bacteria can secrete tumor-boosting metabolites like secondary bile acids, whereas some other species can generate tumor-suppressing metabolites such as SCFAs (141, 142). Echoing previously demonstrated anti-inflammation significance of SCFAs, substantial epidemiological data established that increased incidence of inflammatory diseases and cancer was linked to subjects with diets poor in SCFAs or decreased concentration of fecal SCFAs, typically for gastric and breast cancers (143–145). SCFAs in the gut and other organs can extensively reduce carcinogenesis as well as prevent and treat gastrointestinal and lung cancers, by inhibiting cell growth and migration, suppressing histone deacetylase, and inducing apoptosis (146–148). Specifically, Ohara et al. reported that increased CRC risk correlated tightly with altered gut microbiota, reduced output of SCFAs, and worse inflammation state (149). Therefore, increasing SCFAs production *via* regulation of gut microbiota should have a bright future in anti-cancer therapy.

Other than SCFAs, evidences also indicate that low-dose aspirin could reduce the risk of tumorigenesis, particularly in CRC. As an inhibitor of cyclooxygenase (COX)-1, aspirin exerts its significant chemo-preventive effects in CRC through inhibition of NF-κB dependent pathways, Wnt/β-catenin signaling, and additional COX proteins acetylation (150, 151). The notable anti-tumor effects of aspirin have been observed by reduction in platelet activation, inhibition of tumor angiogenesis, and decrease of pro-inflammatory agents (152). In addition, low-dose aspirin could reduce metastasis in colon cancer, mainly due to its effective inhibition of prostaglandin E2 (PGE2) formation and platelet-tumor cell aggregation (153). Different from aspirin and other modulators of NF-κB, SCFAs could not only inhibit NF-κB signaling pathway but also promote gut microbial ecology and enhance intestinal integrity, further fighting against inflammation and reducing tumorigenesis through multiple pathways. Moreover, as SCFAs can be supplemented by dietary interventions, they were considered to be safer and more obtainable. However, aspirin can cause several severe adverse effects, such as gastrointestinal hemorrhage, intracranial bleeding, and hypersensitivity reactions (154, 155). Even aspirin in a low dose can lead to bleeding in diabetics (156). These advantages push SCFAs to be potential agents to lower cancer risk.

CONCLUSION

Low levels of SCFAs, fermented by gut microbiota, have been linked with DM-related intestinal barrier dysfunction, gut dysbiosis, and aggravated inflammation. Mostly diabetic patients exhibited higher incidence of various tumors. In addition, an inverse relationship between decreased production of SCFAs and increased risk of cancer has also been discovered, showing that SCFAs have the potential of associating DM with tumorigenesis. Accordingly, diets high in fiber and transferring fecal microbiota, aiming at increasing SCFAs production or strains of SCFA-producing species, can attenuate the progression of inflammatory disorders by altering gut microbiota composition and suppressing inflammation (157), which may provide a new paradigm in cancer prevention and treatment in diabetes. However, the effectiveness and security of utilizing SCFAs to dampen inflammation and decrease the incidence and mortality of tumors in diabetic individuals require more research to confirm. Immediate evidences are still needed to validate and recognize the favorable influences of SCFAs as a capable regulator of cancer-related inflammation in DM. Efforts of collaboration encompassing metabolism, microbiology, immunology, oncology, and dietotherapy will define routes of administration and dosage to obtain the optimum benefits of SCFAs as an anti-inflammatory and anticarcinogenic soldier in DM.

AUTHOR CONTRIBUTIONS

QY and JO wrote the first draft of the manuscript. FS provided critical revision of the manuscript. JY conceived and designed the manuscript. All authors contributed to the article and approved the submitted version

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A Comparative Interrupted Times Series on the Health Impact of Probiotic Yogurt Consumption Among School Children From Three to Six Years Old in Southwest Uganda

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Introduction: Following a school milk feeding program in Southwest Uganda, we initiated a probiotic yogurt school feeding program in the same region in 2018. In order to investigate the potential health benefits from probiotic yogurt we conducted an observational study, where we compared the effect of the consumption of locally produced probiotic yogurt containing *Lactobacillus rhamnosus* yoba 2012 to milk in pre-primary schoolchildren from different schools on the occurrence of respiratory tract infections (common cold) and skin infections (e.g., tinea capitis).

Method: A comparative interrupted time series over a period of 3 weeks of baseline followed by 9 weeks of 100 ml of probiotic yogurt or milk consumption for 5 days per week. In total 584 children attending five different schools were followed during consumption of probiotic yogurt and 532 children attending five other schools during consumption of milk. Incidences of respiratory tract infection symptoms and skin infection symptoms, changes in anthropometric indicators and absenteeism were recorded.

Results: Over the course of the study period the incidence rate for common cold symptoms decreased faster in the yogurt group than in the milk group ($p = 0.09$) resulting in a final RR of 0.85 (95% CI: 0.5–1.4) at the end of the observational period. The incidence rate of skin infection related symptoms also reduced faster in the yogurt group compared to the milk group ($p < 0.0001$) resulting in a relative risk factor (RR) of 0.6 (CI: 0.4–0.9) at the end of the observational period. Anthropometric indicators and level of absenteeism did not show significant differences between yogurt and milk.

Conclusion: Notwithstanding the observed positive trend and effect of probiotic yogurt on the incidences of common cold and skin infections, respectively, we consider the results of this comparative interrupted time series inconclusive due to differences in the

recorded health parameters between the probiotic yogurt and milk control groups at base line, and fluctuations over the course of the intervention period. An improved study design, with more uniform study groups, a longer intervention period and a third control group without yogurt or milk is required to draw definitive conclusions.

Keywords: lactobacillus rhamnosus yoba 2012, yogurt, milk, respiratory tract infection, common cold, tinea capitis, school feeding program, probiotics

INTRODUCTION

Disease Incidences

Children in developing countries such as Uganda are at high risk of morbidity due to common childhood diseases. The Ugandan National Demographic and Health Survey indicated that in the Southwestern region of the country, 14% of the children below 5 years of age had suffered from diarrhea, and 11% from Respiratory Tract Infections (RTIs) (1). *Streptococcus pneumonia*-related RTIs together with other lower respiratory tract infections occur in 3% of all children younger than 5 years old in Africa (2) and are the leading cause of child mortality in Uganda. They are responsible for 23% of the deaths of children between 1 and 59 months of age (3). Tinea capitis, a fungal infection, usually caused by *Trichophyton* or *Microsporum* species on the scalp, is a common skin condition among children 2–11 years (4). Tinea capitis is found worldwide, but is more common among children living in crowded households and under poor sanitary conditions, as is commonly found in developing countries such as Uganda (4, 5). National statistics on the incidence of this disease in Uganda are not available. Finally, in the Ankole Region in Southwest Uganda, 29% of the children below 5 years old have been found to be stunted, and 2% is wasted (6). Aflatoxins, carcinogenic substances produced by molds in poorly stored foods, have been proposed as a precursor for stunting (7). Aflatoxins are found in alarmingly high levels in commonly consumed foods in Uganda, most especially in ground nuts and maize (8).

Milk School Feeding Program

The World Health Organization (WHO) recommends the intake of 0.66 g protein/kg body weight per day, as protein is the best source of amino-acids (9). Like all developing countries, the diet in Uganda is starch-dominated, and hence many people do not meet the daily recommended intake of protein (10). Milk is an excellent source of protein with high bioavailability (11). Facing the paradox of poor child growth indicators vs. the high production of milk in the southwestern region, SNV (The Netherlands Development Organization) designed a program to promote the consumption of milk in schools as part of a larger developmental project in the dairy sector in this region, called The Inclusive Dairy Enterprise Project (TIDE). Under this program, primary schools in seven districts in Southwest Uganda that are part of the Ankole sub-region (Kiruhura, Lyantonde, Bushenyi, Sheema, Isingiro, Ntungamo, and Mbarara) have been participating. School leaders and parents have been sensitized about the health benefits of regular milk consumption for child development and general well-being, and encouraged to pay an additional school fee for the milk. For ∼5.5 USD, a child receives 100 ml of milk for 5 days per week during a school term of 12 weeks. Raw milk is delivered and boiled at the school premises as a component of maize porridge, after which it is consumed by the children in the form of a hot beverage. After 4 years of implementation, ∼300,000 primary and pre-primary school children have been enrolled in the program.

Yogurt School Feeding Program

Following the success of the TIDE school milk program, SNV and the Yoba for Life foundation designed a similar program including locally produced probiotic yogurt instead of milk. The rationale for shifting from milk to probiotic yogurt comes from the assumption that the probiotic bacteria, especially *Lactobacillus rhamnosus* yoba 2012, the generic version of *L. rhamnosus* GG, could boost the immunity and alleviate infections and diseases that are frequently occurring in young children, including diarrhea, common cold, allergies, skin conditions and growth retardation.

The Probiotic Bacterium *Lactobacillus rhamnosus* GG

The bacterium *L. rhamnosus* GG is the world's best documented probiotic with a number of proven health benefits (12), including the prevention and reduction of diarrhea (13), common cold (14), allergies and skin conditions (15). The probiotic strain used in the locally produced probiotic yogurt in Uganda is a generic variant of *L. rhamnosus* GG, called *Lactobacillus rhamnosus* yoba 2012 (16). Over 30 studies have been conducted on the effect of probiotics on children attending day care centers (17, 18), the majority of them looking at the incidence and duration of gastrointestinal infections and respiratory tract infections (RTIs). No adverse effects of the consumption of *L. rhamnosus* GG have been reported in children to date. An overview of the studies that use *L. rhamnosus* species is presented in **Supplementary Table 1**. In the current study, we specifically look at the incidence of common cold and skin infections. Common cold is the most common form of respiratory tract infection.

With regard to skin infections, a number of studies have been conducted on the incidence of tinea capitis among primary school children in various African countries. For example, Ayaya et al. (5) found an incidence of 33% among children attending a primary school in Kenya, Chepchirchir et al. (19) found 11% among eight primary schools in urban slums in Kenya, and Ngwogu and Otokunefor (20) found an incidence of 20% among children attending primary schools in Nigeria. Two *in vitro* studies showed a strong inhibitory effect of lactic acid bacteria

species on the growth of the fungal species *Microsporum* and *Trichophyton,* which are responsible for tinea capitis (21, 22). The mechanism of action presumably comes from the production of anti-fungal compounds. To date no studies have been performed on effects of probiotic intake on tinea capitis in humans resulting from a presumed immune modulatory mechanism.

In spite of the large body of evidence showing the ability of *L. rhamnosus* GG to prevent and reduce the above-mentioned conditions, the efficacy of *Lactobacillus rhamnosus* has not been studied in the population of Ugandan children. Yet in Uganda, there is an established and sustainable supply of *Lactobacillus rhamnosus* yoba 2012 through the local production of probiotic yogurt throughout the country by the use of the "yoba" starter culture (23, 24), as well as through the program to promote its consumption among children in pre-primary schools. These practices prompted us to study the health benefits of the probiotic *L. rhamnosus* for this specific population group.

Experimental proof for the efficacy of *L. rhamnosus* GG has been described in many studies, mostly in the form of probiotic supplements. However, this is the first time that we evaluate health effects of the consumption of *L. rhamnosus* yoba 2012 in children consuming locally produced probiotic yogurt containing *L. rhamnosus* yoba 2012 and *S. thermophilus* C106. Only children from whom the parents had agreed to pay for yogurt or milk consumption as ongoing practice participated in this study.

Local Production of Probiotic Yogurt

Since 2012 the local production of probiotic yogurt has been promoted in Uganda, resulting in a production network of more than 130 small scale yogurt production units (24). The yogurt program is similar to the school milk program and has been implemented in the same area, but targets specifically pre-primary schools. The cost of taking 100 ml of yogurt for 5 days per week is 6.50 USD per school term. The program intends to simultaneously support the health of young children, as well as creating a market for the locally produced yogurt, thereby facilitating women empowerment, increasing employment opportunities and raising household incomes (24, 25). Since the beginning of the program in 2018, over 20,000 pre-primary children have been enrolled in the probiotic yogurt school feeding program.

METHODS

Subjects and Design

This study followed a comparative interrupted time series design (CITS), carried out over a period of 12 weeks, and included 1,116 children aged 3 to 6 years old. The study compared health related parameters of children consuming 100 ml of milk per day to children consuming 100 ml of probiotic yogurt containing *Lactobacillus rhamnosus* yoba 2012 and *Streptococcus thermophilus* C106 per day, for 5 days per week during 3 weeks of baseline and 9 weeks of dairy consumption. Five schools participated in the milk group (Faith Memorial Nursery and Primary School Bushenyi, Queen and King Nursery and Primary School Itojo Ntungamo, St. Eliza Excell Nursery and Primary School Isingiro, Jireh Junior School Ishaka Bushenyi, St. Francis Nursery and Primary School Lyantonde and Primary School Isingiro Itojo, with 171,132, 85, 74, and 70 children, respectively, total $N = 532$) and five schools participated in the yogurt group (Blue Sight Primary School Kabwohe Sheema, Hanny Nursery and Primary School Isingiro, Mbarara Progressive Nursery and Primary School, BDA Nursery and Primary School Ishaka Bushenyi, Itojo Nursery and Primary School Itojo Ntungamo, with 177, 139, 115, 77, and 76 children, respectively, total $N = 584$).

Selection of schools was based on enrolment of the school in the yogurt or milk program as promoted by SNV and the Yoba for Life Foundation. This implied that the parents agreed to pay for the milk or yogurt consumption of their children, not only for the duration of the study, but as an ongoing practice. This selection criterion ensured that children will profit from long-term benefits from yogurt or milk consumption. Before participation in the participating study, written consent was obtained from parents or caregivers of all participating children.

Intake of Dairy Products

Milk was supplied by local farmers or dairy cooperatives who were identified by SNV/TIDE in collaboration with the school. The milk was delivered to the schools as fresh milk in aluminum milk cans, and tested with milk quality tests, as described previously (26). The milk was boiled at the school premises together with water and maize flour, into a maize porridge. Each child in the study consumed 400 ml maize porridge per day, which contained 100 ml milk.

The yogurt for the five schools was produced by five local producers (Nunu probiotic yogurt, Mbarara; Tiana Foods, Mbarara; Blessed Choice, Ntungamo; Rwembogo, Isingiro; K-yoba, Sheema). The yogurt was made using a starter culture containing *Lactobacillus rhamnosus* yoba 2012 and *Streptococcus thermophilus* C106 (26) and contained 5% (w/v) sugar and 0.1% (w/v) artificial flavor (strawberry or vanilla). The yogurt was packed in small, specifically designed polythene bags of 100 ml (**Supplementary Material Picture 1**), as is a common practice in Uganda. The yogurt was delivered at the schools during weekdays at the agreed delivery time. The drinking yogurt was consumed by the children with a straw. Waste bins were provided to the schools for plastic waste collection. On a daily basis, the quality of the yogurt was monitored by organoleptic tests (taste and appearance) before being served to the children. In collaboration with the local authorities (Dairy Development Authority), samples of the five producers were taken twice during the study in order to test for the presence of pathogenic bacteria.

In accordance with the cell count experiments as described previously (23, 26), a daily serving size of 100 ml of the probiotic yogurt contains $\sim 5 \times 10^9$ CFU *L. rhamnosus* yoba 2012 and 1×10^{11} CFU *S. thermophilus* C106 per day.

Parent Questionnaires

Three times during the study, in week 1 and 2, 5 and 6 and 11 and 12 a questionnaire (**Supplementary Text 1**) was conducted among the parents of the participating children. The objectives

of this questionnaire were to collect data about (1) the socio-demographic characteristics of the children that might affect the study outcomes, (2) the diet of the child, specifically with regards to the intake of dairy products and fermented foods, (3) the health status of the child for cross checking and complementing the data that were collected by the nurses (see below).

Dietary information was evaluated and quantified using the Dietary Diversity Score (DDS) tool and assessed the variety of foods that a household accesses and consumes on an average day, as reported by Swindale and Bilinsky (27). Food diversity was scored between 0 and 12, based on a recall of food eaten in the last 24 h from a maximum of 12 different food groups.

Absenteeism

Teachers in every school kept daily track of child absenteeism by using specifically designed attendance lists.

Monitoring of Common Cold Symptoms and Skin Conditions

Five nurses were engaged in the study and visited the schools 5 days per week to monitor the incidence of skin infections and common cold related symptoms among the children. The school nurses observed every child individually, thereby focusing on detecting signs of common cold, including cough, runny nose, blocked nose, sore throat, fever, headache, malaise, loss of appetite, as well as skin conditions, including any type of irregularity, infection, wound or rash on the skin. In case of absenteeism, the teacher was asked to try to reach the parent of the absent child by phone, to inquire for the reason for absence, which was subsequently recorded by the nurse.

All nurses were equipped with tablets that contained a software application (app) (Kenga Mobile, OMNI-Tech Ltd, 2018) that was specifically developed for this study (downloadable from Google Play Store). The app facilitated the reporting on skin infections and common cold related symptoms for each individual child (see **Supplementary Text 1**). For skin infections, the type and the part of the body that was affected, were specified, and a picture of the affected body part was taken as part of the app questionnaire. For common cold, the specific symptoms were indicated via the app. No therapeutic actions were supposed to be taken by the nurses, except in case of emergencies.

Anthropometric Indicators

Anthropometric data were taken four times during the study (**Figure 1**), including weight, as measured on a digital weighing scale (Casa CEGS01, South-Africa) and height as measured with a height rod with stand (Fazzini S225, Italy). All equipment was calibrated, and the nurses had been collectively trained on its use and were individually coached and monitored during their activities. Training of the nurses had been performed according to the WHO guidelines for standardized measurement procedures (28).

Data Collection and Analysis

All data was collected through a specifically designed mobile software application, mentioned before, from where it was uploaded onto a protected online platform with access only for the researchers. All children were registered in the app. The app contained three different "forms" (see **Supplementary Text 1**), which were linked to each child: 1. a form for parent questionnaires, 2. a form for reporting incidences of diseases, and 3. a form to report anthropometric measures. From the online data collection platform, data was exported to Excel for further analysis.

Propensity Score Weighting

For analysis of the incidence of common cold, skin infections and absenteeism, the percentage of the total study group that was sick, was considered as the unit of analysis. Propensity score weighting was used to correct for differences in baseline characteristics of the children. Propensity scores were calculated based on socio-demographic characteristics as obtained through the parents' interviews at baseline. Weighting was done based on both the standardized mortality ratio weighted (SMR) method, as described by Kurth et al. (29). The incidence of common cold, skin infections and absenteeism were treated as individual interrupted time series, and analyzed according to the segmented regression methods, as described by Wagner et al. (30) and Linden and Adams (31). Model fitted trend lines were obtained through (1) three median smoothing (datapoint replaced by median of the data point, the preceding datapoint and the following datapoint, (2) hanning (0.25 xprevious data point + 0.5 xdata point + 0.25 xfollowing data point), and (3) skip mean (average of the previous and the following

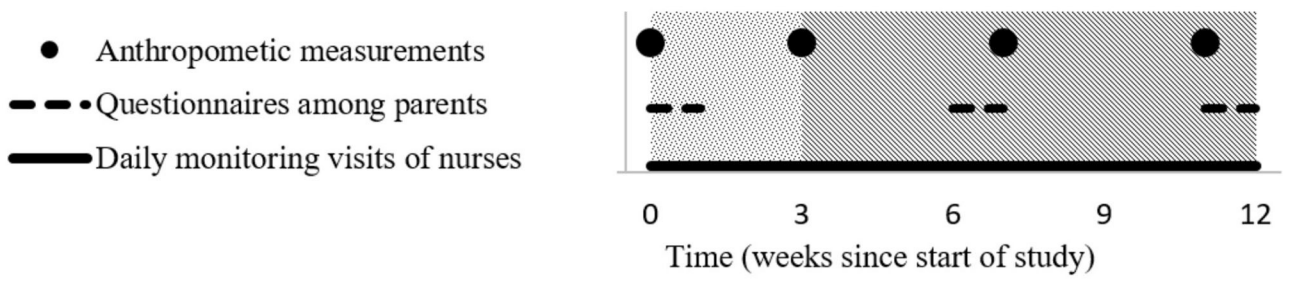

FIGURE 1 | Timeline indicating the moments of different data collection. Dotted area = baseline. Diagonal striped area = milk/yogurt consumption period.

data point). All hypothesis tests used a significance level (α) of 0.05. All analyses were done using Microsoft Office Excel 2019.

Analysis of Anthropometrics

Anthropometric records were evaluated with software provided by the Word Health Organization (WHO Anthro version 3.2.2 and WHO AnthroPlus version 1.0.4) (32, 33). With this software package, measures of weight and height were assessed in reference to the WHO standard growth curves, and expressed as Z-scores, which are the number of standard deviations away from the standard value as obtained from the standard growth curves (34, 35).

RESULTS

Socio-Demographic Characteristics and Propensity Score

Socio-demographic characteristics of the children in the yogurt and milk group were very similar in the average age of the household head, the composition of the household, sanitary conditions, and methods of preparing drinking water (**Table 1**). However, differences were pronounced in the proportion of female-headed households (widows or single mothers), level of education of the parents, source of water (e.g., piped water or well) and school fees paid. More specifically, on average children in the yogurt group belonged to higher income families compared to children in the milk group. Hence, a

TABLE 1 | Household characteristics of the yogurt group ($n = 577$) and milk group ($n = 492$), collected during the baseline questionnaire in the first 2 weeks of the study.

Category		Original		SMR-weighted	
		Milk	Yogurt	Milk	Yogurt
Gender	Boys	51%	52%	55%	52%
	Girls	49%	48%	45%	48%
Average age child		4.84	4.79	5.14	4.79
Household	Male headed	80%	89%	87%	89%
	Female headed	20%	11%	13%	11%
Average age household head		36.37	36.60	38.15	36.60
People in household		5.32	5.53	5.66	5.53
Females $\leq$ 5 years		0.85	0.85	0.89	0.85
Males $\leq$ 5 years		0.84	0.87	0.95	0.87
Females 6–13 years		0.50	0.57	0.45	0.57
Males 6–13 years		0.60	0.66	0.64	0.66
Females 14–59 years		1.32	1.26	1.40	1.26
Males 14–59 years		1.06	1.09	1.14	1.09
Females $\geq$ 60 years		0.09	0.11	0.10	0.11
Males $\geq$ 60 years		0.05	0.13	0.08	0.13
Education level head	No formal education	4%	2%	2%	2%
	Primary	31%	18%	24%	18%
	Secondary	29%	33%	27%	33%
	Tertiary	37%	46%	47%	46%
	Toilet	98%	98%	98%	98%
Sanitary condition	No toilet	2%	2%	2%	2%
	Protected well or spring	12%	5%	8%	5%
Source of water	Borehole	5%	3%	2%	3%
	Open spring or well	17%	9%	9%	9%
	Surface water	1%	0%	0%	0%
	Rain water	2%	3%	1%	3%
	Piped water	63%	79%	81%	79%
Drinking water	Boil water	99%	100%	98%	100%
	Let it stand and settle	1%	0%	1%	0%
Food taken in the last 24 h	Fermented cereal (bushera)–baseline	26%	8%	9%	8%
	Fermented cereal (bushera)–midline	12%	14%		
	Fermented cereal (bushera)–end line	10%	11%		
	Milk	72%	58%	68%	58%
	Fermented milk	3%	4%	3%	4%
School fees		127	196	127	196

propensity score that was calculated based on socio-demographic characteristics as obtained through the parents' interviews at baseline, was assigned to the study subjects. Next, propensity score weighting was used to correct for differences in baseline characteristics of the children. As a result, the similarity of socio-demographic characteristics in the control and yogurt group improved notably, as is shown in **Table 1**.

Dietary Indicators

Dietary diversity scores (DDS) calculated at baseline, midline and end line for the yogurt group were 6.4, 6.5, 6.8, respectively, and for the milk group 6.2, 6.5, 6.5, respectively. The DDS in the yogurt group was slightly higher, possibly as a result of a relatively higher wealth status, as mentioned before. On an average day before the study started, 58% of the children in the yogurt group and 72% of the children in the milk group would consume dairy products (**Table 1**). As a result of the yogurt or milk consumption, this increased to 88% in the yogurt and 91% in the milk group (during weekdays every child consumed a milk product, but on Saturday and Sunday this depended on household choices). The increased consumption of milk products explains for a large part the slight increase of DDS in midline and end line as compared to baseline.

At household level, on an average day during the study, 11% of the children in the yogurt group and 16% of the children in the milk group consumed a fermented cereal porridge called bushera (36). In addition, on an average day during baseline, 4% of the children in the yogurt group and 3% of the children in the milk group consumed fermented milk products (locally fermented milk or regular yogurt).

Evaluation of Skin Conditions and Common Cold Symptoms

Data from skin condition observation and common cold collected during the 5 weeks of study by the locally employed nurses at the ten different schools, are presented in **Supplementary Figures 1A–F**.

Skin Infections

During the 12 weeks of the study, 30% of the children in the yogurt group and 35% of the children in the milk group were found to have a pathological skin condition at some point in time. The most common identified skin conditions occurred on the scalp, i.e., tinea capitis (fungal infection) and folliculitis (bacterial infection of hair follicles). Usually, the children suffered from a combination of both conditions. These two scalp conditions together accounted for 89 and 90% of all the reported skin conditions in the yogurt and milk group, respectively.

During the 3 weeks of baseline measurement, the incidence of skin conditions was found to be higher in the yogurt group compared to the milk group, with averages of 7 and 3%, respectively (**Figure 2A**). In the subsequent 3 weeks, which were the first 3 weeks after the start of intake of yogurt or milk, the incidence of skin conditions rose substantially in both groups. However, this rise is stronger in the milk schools compared to the yogurt schools group. Furthermore, after a peak at 6 weeks, the incidence of skin conditions in the yogurt group decreased, while the incidence in the milk group continued to fluctuate with an average decrease over the remaining 4 weeks of (**Figure 2A**).

The difference in the relative incidence of skin infection symptoms between the yogurt group and the milk group, and the 95% confidence intervals, are shown in **Figure 2B**. Linear regression is used to analyse trends, see **Supplementary Table 2**. After the start of yogurt or milk consumption the incidence of skin infections showed a significant relative decrease over time (P-value trendline < 0.0001) in the yogurt group compared to the milk group. This trendline results in a final relative risk (RR) of 0.6 (CI: 0.4–0.9) for the incidence of skin infections after 8 weeks of probiotic yogurt consumption compared to milk consumption. However, as different levels of skin infections were observed in seven out of the ten measurement points during the baseline period for the two groups, care should be taken when interpreting these results.

For a more detailed evaluation of the data, the measurement points during consumption period of yogurt or milk were split-up into three time periods of 13 measurement points, each representing 13 weekdays, as shown by the gray vertical lines in **Figure 2A**. The average number of days with observed skin infection symptoms per child for each of these time periods is shown in **Table 2**. In the first time period, incidence and average duration of skin infections were still increasing, probably as a result of cross-infection in the classrooms. However, the relative increase in the yogurt group during this time period was less compared to the milk group. In the second time period, the increase in infections is halted in both groups and in the third time period the average duration of observed skin infections decreased with 26% in the yogurt group compared to a much smaller decrease of 6% in the milk group. These findings are further supported by the analysis of the number of children without reported skin infection symptoms in the same periods of 13 days (**Table 2**). While in the second time period an increase in the number of children with skin infection symptoms was observed, during the last period the number of children without symptoms increased with 5% in the milk group and 13% in the yogurt group. The observed amelioration suggests a potential beneficial effect of probiotics, which increases over time.

Common Cold

The incidence of the common cold started to rise immediately when the children went back to school. As soon as the yogurt or milk consumption started there was a trend of decreasing incidence of common cold in the yogurt group as well as in the milk group. However, this trend was slightly stronger in the yogurt group (**Figure 3A**).

The difference and the 95% confidence intervals in the relative incidence of common cold in the yogurt group over the milk group is shown in **Figure 3B**. Linear regression was used to analyse trends, see **Supplementary Table 2**. Following the base line period, there is a slight decrease over time in the number of children suffering from common cold in the yogurt group compared to the milk group (P-value trend line $= 0.09$). This end line results in a final RR of 0.85 (CI: 0.5–.4) for the incidence of common cold after 8 weeks of probiotic yogurt consumption.

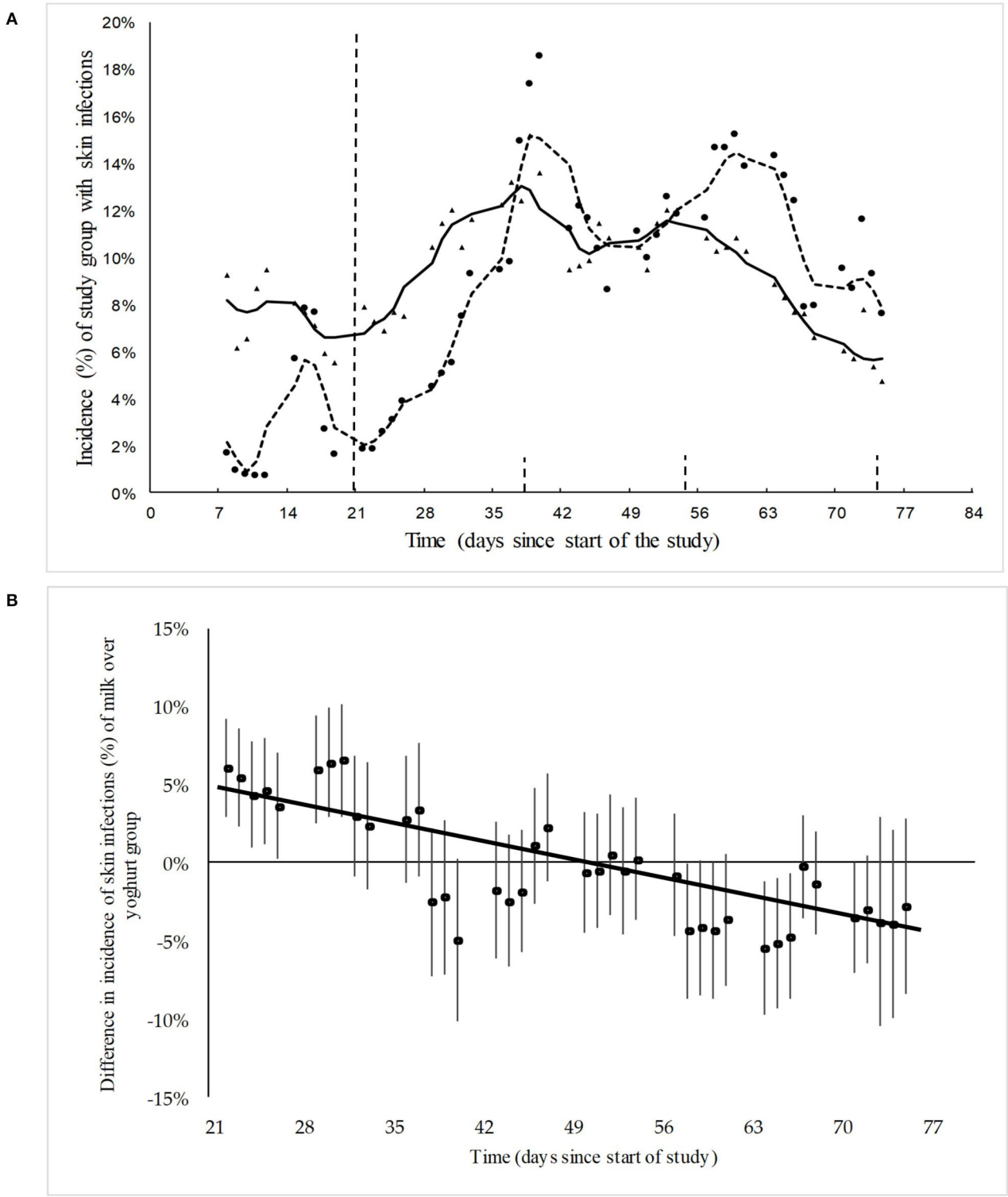

FIGURE 2 | (A) The actual and model-fitted SMR-weighted incidence of skin infections in the yogurt group ($n = 507$) group and milk group ($n = 287$) between day 7 and day 73, expressed as a percentage of the total children under observation. Δ = data points yogurt group; solid line = trend yogurt group; • Data points milk group; dotted line = trend milk group. The baseline is from day 1 to day 21 and the yogurt/milk consumption period is from day 22 to day 84. **(B)** The actual and model-fitted difference and the 95% confidence intervals in the relative incidence of skin infections in the yogurt group over the milk group as recorded after baseline period.

TABLE 2 | Comparative analysis of parameters for skin infection and common cold related symptoms per time period of 13 days of measurements during milk and yogurt consumption (a total period of 39 days of measurements in a period of 8 weeks). Note that a negative value means a reduction.

Parameter	Period 1	Period 2	Period 3	Difference		Relative difference		*P*-value
				2-1	3-2	2-1	3-2	
Average number of days of skin infection per child (milk group)	0.75	1.42	1.34	0.67	0.08	89%	−6%	0.30
Average number of days of skin infection per child (yogurt group)	1.34	1.47	1.08	0.13	0.38	9%	−26%	0.0004
Number of children without skin infection (milk group)	222 (77%)	203 (71%)	214 (75%)	−19	11	−9%	5%	0.30
Number of children without skin infection (yogurt group)	402 (79%)	382 (75%)	430 (85%)	−20	48	−5%	13%	0.0002
Average number of days of common cold per child (milk group)	1.16	0.82	0.82	0.34	0.00	−29%	0%	0.71
Average number of days of common cold per child (yogurt group)	1.26	1.08	0.75	0.18	0.33	−14%	−31%	0.097
Number of children without common cold (milk group)	332 (62%)	383 (72%)	379 (71%)	51	−4	15%	−1%	0.79
Number of children without common cold (yogurt group)	395 (68%)	424 (73%)	438 (75%)	29	14	17%	3%	0.35

Group sizes for skin infections measurements included n = 507 for the yogurt group, and n = 287 for the milk group, and for common cold measurements n = 584 for the yogurt group, and n = 532 for the milk group.

Also for this study outcome, the measurement points during consumption of yogurt or milk were split-up up into three time periods of 13 measurement points (representing 13 week days), as indicated by the gray vertical lines in **Figure 3A**. The average number of days with common cold symptoms per child for each time period is shown in **Table 2**. Throughout the first and the second time period the incidence and duration of the common cold decreased for both groups. In the last time period, this increase leveled off for the milk group, while it continued to decrease with 31% for the yogurt group. These observations are in agreement with the number of children that have not been reported with common cold symptoms during the three time periods (**Table 2**). More specifically, in the second time period we could still observe an increase in the number of children with common cold symptoms in both groups. However, during the last time period this changed to a small increase (3%) in the number of healthy children in the yogurt group, while the number of children without symptoms reported for the milk group remained around the same level (−1%).

In-depth analysis of the course of different symptoms of underlying common cold shows that the development of rhinitis is similar to the general course of common cold, while for cough we see a sharp drop in symptoms in the yogurt group as soon as the consumption started. It should be noted that this observed reduction in the cough symptoms was dominated by findings of only one of the five yogurt consumption schools (**Supplementary Figures 2A,B**).

Absenteeism

The average absenteeism during week two and three of the baseline period was 3.4% (SD 0.86%) in the yogurt group, and 3.3% (SD 1.1%) in the milk group (Note: the first week was not considered representative as many children had not yet returned after holidays). The average absenteeism during the yogurt or milk consumption period dropped to 2.7% (SD 1.2%) in the yogurt group and 2.3% (SD 0.80%) in the milk group.

Anthropometric Indicators

The average age, weight and height of the children in both groups at the four measurement points have been indicated at **Supplementary Table 3**. This table also shows the SMR-weighted average scores for the height-for-age (HAZ), weight-for-age (WAZ) and Body Mass Index-for-age (BAZ) at the four different measurement points during the study. Children in both groups have average lengths and weights, although the average HAZ and WAZ values are slightly below, and BAZ values are slightly above the WHO standards. The children in the yogurt group were slightly shorter for their age compared to the children in the milk group. In this study we found an overall rate for stunting and wasting of 9 and 3%, respectively. Whereas, the rate of stunting is far below the 29% that the Uganda Bureau of Statistics found in the Demographic and Health survey of 2016 (6), the rate of wasting is similar to what has been reported in the survey.

From the results of the current study it can be concluded that the consumption of either 100 ml milk or 100 ml probiotic yogurt per day for 5 days per week, does not have a measurable effect on the growth parameters of children within the relatively short period of 8 to 9 weeks (*P*-value WAZ and BAZ end-line difference yogurt and milk are 0.34 and 0.57, respectively). However, it is remarkable that in both groups the growth parameter scores decreased over the study period for yogurt and milk (**Supplementary Tables 3, 4**), suggesting that the during the school term the average energy expenditure was higher than the average energy intake.

DISCUSSION

Strengths of This Study

The comparative interrupted time series (CITS) was conducted as part of an ongoing developmental program that introduced milk and yogurt in schools, as outlined in the introduction. This is the first time that effects of the consumption of probiotic yogurt containing *L. rhamnosus* yoba 2012 are studied in children attending pre-primary schools on the African continent. Only

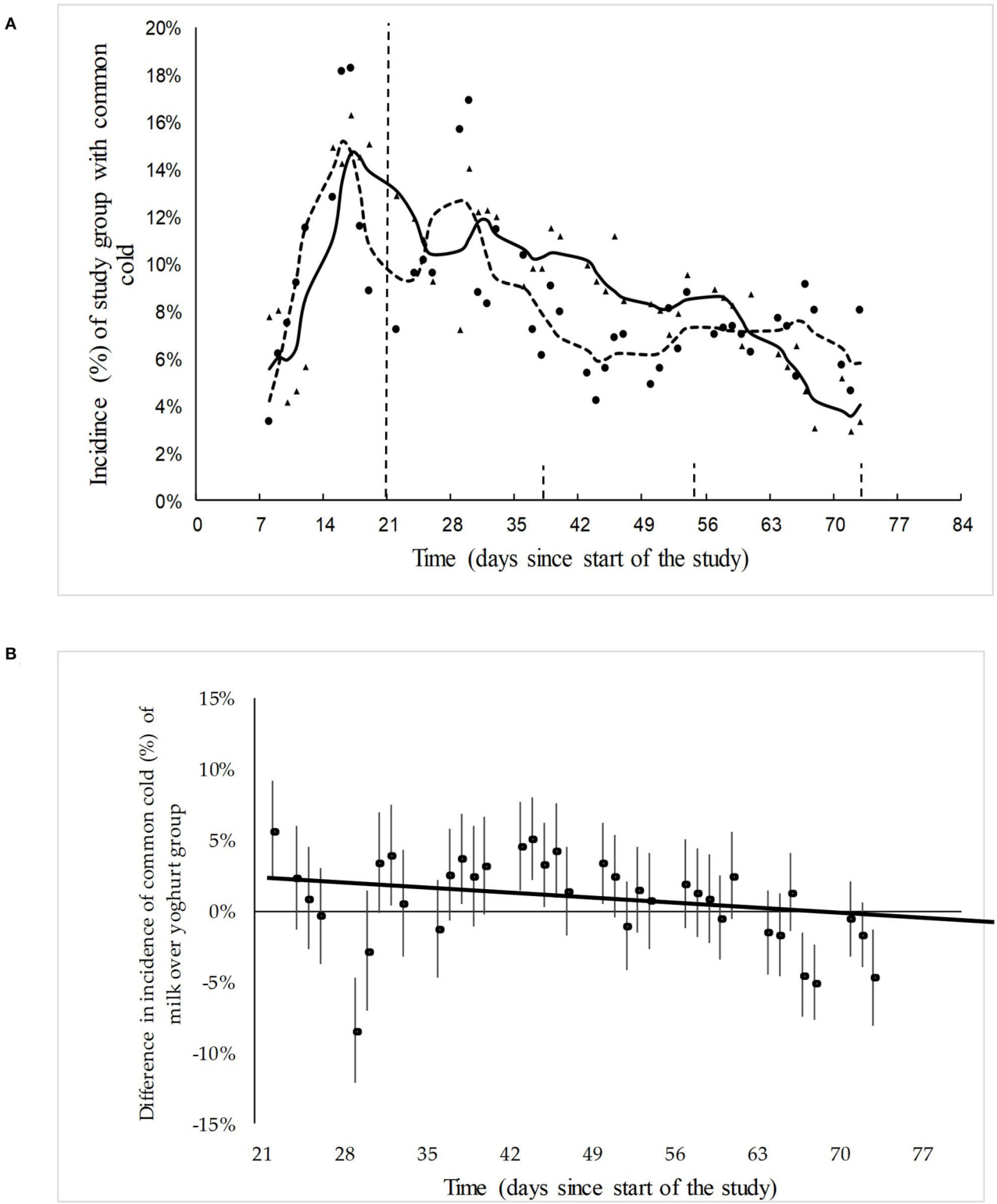

FIGURE 3 | (A) The actual and model-fitted SMR-weighted incidence of skin infections in the yogurt group ($n = 584$) group and milk group ($n = 532$) between day 7 and day 73, expressed as a percentage of the total children under observation. Δ = data points yogurt group; solid line = trend yogurt group; • Data points milk group; dotted line = trend milk group. The baseline is from day 1 to day 21 and the yogurt/milk consumption period is from day 22 to day 84. **(B)** The actual and model-fitted difference and the 95% confidence intervals in the relative incidence of common cold in the yogurt group over the milk group during the dairy consumption period.

children were recruited in case the parents agreed to pay for yogurt or milk consumption as ongoing practice.

In contrast to many intervention studies, the present study resembled a real-life situation of primary schools in Southwest Uganda where probiotic yogurt was already served. In order to allow baseline measurement for this study, ten new schools were recruited to start with the yogurt or milk school feeding program. During the study there was minimal distortion of daily practices at the participating schools. In addition, no dietary or behavior guidelines were given to the participating children and their parents, except for the daily consumption of 100 ml of probiotic yogurt or milk.

The comparative interrupted time series included a relatively high frequency (almost daily) of data collection over the course of 10 weeks. Compared to most nutritional intervention studies, where data is only collected at beginning, end, and sometimes mid-point, the CITS allows for a more precise observation of trends during baseline and the consumption phase of milk or yogurt, such as the occurrence of cross infections between children during the first weeks after the beginning of the school term.

The yogurt was locally made by producers who were not only recruited to produce the product for this study, but who were already supplying the probiotic yogurt to neighboring schools. The producers received both technical as well as business guidance on a periodic basis, and the quality of the milk and probiotic yogurt products was regularly tested.

In terms of health benefits, the children enrolled in the yogurt school feeding program profit from the multiple benefits from a functional fermented food (37). More specifically, in this yogurt the probiotic *L. rhamnosus* yoba 2012 has propagated in the milk matrix during the fermentation. As a consequence, and contrary to concentrated probiotic dietary supplements, the fermented milk matrix also contains bioactive metabolites like acids and vitamins produced by *L. rhamnosus* and *S. thermophilus* bacteria during milk fermentation.

Limitations of This Study

Although there is a large body of literature pointing to the health benefits of *L. rhamnosus* yoba 2012 (*L. rhamnosus* GG), currently no studies are available on the health benefits of *S. thermophilus* C106. As a consequence, for all results presented above, we cannot exclude the role of *S. thermophilus* C106, or synergistic effects between the two bacteria.

The study was non-randomized, and participation in either the yogurt or milk group depended on the school's and parents' choice to either enroll in the school yogurt or in the school milk program. As the probiotic yogurt program was slightly more expensive, and as yogurt is an unknown product to part of the rural population, the yogurt program was particularly popular among schools where children from higher economic status attended.

This study aimed to investigate the impact of probiotics present in the locally produced probiotic yogurt on the lives of children in Southwest Uganda as part of their regular diet. Hence, children were not advised to change their normal dietary habits. Rather, their dietary habits have been assessed, and used as a parameter for calculation of propensity scores of the children (see section Methods). More specifically, different schools received different school meals in addition to the 100 ml of milk or yogurt. Furthermore, the high level of milk consumption and significant level of consumption of fermented foods of children at baseline and during the subsequent yogurt or milk consumption period, may have reduced the additional impact of the probiotic yogurt. The health benefits studied here may have partially resulted from the consumption of fermented foods during the study period (37).

A challenge related to the duration of the study was set to a school term of 12 weeks. In Uganda, school fees are paid by the parents every school term, and usually parents wait till the last moment to start collecting the fees, which need to be paid in order to get access to the school. Hence, at the beginning of the school term, absenteeism is very high. Furthermore, toward the end of the term, absenteeism again increases, as children leave school early for holidays. Consequently, due to high absence of children in the first week of the study and early start of the holidays in 8 of the 10 schools in the last (twelfth) week of the study, these weeks were excluded from data analysis.

In addition, many schools had put restrictions on the time and duration of the daily visitations of the school nurse. Hence, the nurses were sometimes rushed, which may have caused overlooking of certain incidences of infections or symptoms. This may have contributed to an irregularity in reports of skin conditions or common cold symptoms. For instance, sometimes skin infection symptoms were not continuously indicated, but on separate days within a period of e.g., 10 days. However, in view of the normal development of skin infections (38, 39), it is unlikely that the condition would disappear 1 day and would return the following day. The gaps in reporting could also have been caused by the child being absent, the nurse not attending the school on that particular day, or the hair growth of the child. (Long hair makes the skin condition harder to detect, and children usually go for shaving every 1 or 2 weeks, after which the condition of the head skin becomes much more visible).

In case of missing data on the reporting of skin infections, interpolation of the data points was done before calculating the total incidence of infections. For the subsequent data analysis, it was assumed that gaps smaller than 10 days actually represented one ongoing episode of skin infection. For absence of skin infection indications of more than 10 days, we assumed it concerned two different episodes of skin infection. From all the possible data points that could have been reported, 82% were actually collected. Furthermore, at schools Faith Memorial, Jireh Junior (milk group) and BDA junior (yogurt group) it was noted that the responsible nurse had not recorded occurrences of skin infections at all (**Supplementary Figures 1A,B**). In order to avoid a bias in the data interpretation, the authors decided to exclude these schools in analysis of skin infection symptoms and the number of evaluated children has been adjusted in the corresponding Tables and Figures.

In case of missing data for common cold symptoms, gaps of data for every individual school were closed by linear interpolation of the incidence of common cold on days adjacent to the gap. Next, the average incidence of common cold in either

the yogurt or the milk group was calculated. From all the possible data points that could have been reported, 85% of the data was available, while the remaining 15% was based on interpolation.

Skin Conditions

The incidence rate of *tinea capitis* was in the same range as reported by other authors who conducted studies among African children (5, 19, 20). The increase in skin conditions during the first 6 weeks of the study may be due to the fact that most of these skin conditions are highly transmittable (39), and hence their prevalence increases when children come back to school after holidays. The difference between incidence of skin conditions at baseline in the milk and yogurt group is also similar to differences observed by other authors who reported highly varying incidence rates of tinea capitis between schools in the same region (19). Differences in baseline values between study groups are common in non-randomized studies, but compromise the robustness. The decreasing incidence of skin infections in the yogurt group relative to the milk group at the start of the consumption period (**Figure 2B**), suggests a positive effect of the consumption of probiotic yogurt and corresponds to observations made during a previous pilot study we conducted in Uganda among 245 children (24). With regard to atopic dermatitis, from a review of 13 studies on the incidence of this skin disease among children below 3 years of age, we conclude there is a preventive as well as reducing effect by *L. rhamnosus* GG (40). Also, other studies showed that intake of the probiotic *L. rhamnosus* GG led to reduction of eczema and allergic reactions (15, 41, 42). However, in the current study the overall incidence of atopic dermatitis and eczema was too low, and there may be too many confounding factors in order to draw conclusions about effects of probiotic yogurt on these specific conditions.

Notwithstanding the positive results observed for the probiotic yogurt group vs. the milk group, we would like to refrain from making definitive conclusions, and instead propose follow-up studies with an adjusted study design. Especially, the differences at baseline, the increase of infection at the start of the consumption in both groups, as well as a number of possible confounding factors, including differences in individual diet, behavior and social-economic situation of the participating children and their families, has led to our recommendation to conduct follow-up studies over a longer period of time, in a more uniform background situation, and with additional controls such as a study group without yogurt or milk.

Common Cold

The increase of common cold during the baseline in both control and yogurt group may be explained by its highly infectious nature among children in the classroom. Also, from other studies we know that the risk ratio is 1.9 of contracting common cold for a young child attending day-care, compared to a child kept at home (43). The slightly decreasing incidence of common cold in the yogurt group relative to the milk group during the consumption period (**Figure 3B**) only indicates a trend., It should be noted that previous studies and meta-analyses with different study designs showed more pronounced effects of *L. rhamnosus* GG on different types of respiratory tract infections, possibly through direct antimicrobial effects, improved mucosal barrier function and immunomodulating activity (14, 44–51), see also **Supplementary Table 1**. The reduction of common cold symptoms after the start of the consumption of milk and yogurt in both groups may be explained by seasonal influences or by an immune-boosting effect of consumption of milk and yogurt, e.g, as a result of its nutritional value of micro- and macro nutrients. Previous research has shown that milk contains components including fatty acids, lauric acid and zinc that have antiviral properties or play a role in immune function (52), as well as bioactive peptides (53–55). As reported above for skin diseases, also for the incidences of common cold, we would like to refrain from making definitive conclusions, and we rather propose follow-up studies with an improved study design as summarized above.

Absenteeism

An increase in school attendance as a result of school feeding programs has been reported by several other authors. Studies among low-income or food-insecure children in North-Uganda (56), Ghana (57), and South-Ethiopia (58) showed increases in school attendance of 13 percent points, 15 and 13%, respectively, as a result of offering meals at school. A study among children in Kenya with a baseline attendance of 95%, still showed an increase in attendance of 1–4 percentage points for different school meals provided (59). In the present CIRS, the observed school attendance was already relatively high and all schools had already implemented school feeding programs, in addition to the supplementation of milk or yogurt. Consequently, we assume that the measured effect of the provision of dairy products on school attendance was almost negligible.

Anthropometric Indicators

The anthropometric indicator WAZ slightly decreased during the study. As a result, in both groups we also observed during the study period a reduction in BAZ value. We hypothesize that children eat less or use more energy when they are at school compared to when they are at home during the holidays. This observation argues for additional school feeding programs with preferably higher quantities of food than the 100 ml of daily milk or yogurt servings provided in the present study.

A study with school milk in Iran did not find significant changes in anthropometric indicators for boys after 3 months of intervention (60). Only one study in New Guinea found more growth in children supplemented with milk compared to the control group. However, the sample size in this study was extremely small (61). A study in Kenya running for a period of 2 years indicated improved growth in children below 6 years of age, and in stunted children (62).

A retrospective study showed a correlation between higher milk consumption and higher values for BMI and height for 4 year old children (63). Similarly, a retrospective cohort study of 12,376 children in South East Asia indicated significant lower levels of stunting, underweight, vitamin A and vitamin D deficiency for children who would consume dairy on a daily basis, compared to those who did not consume dairy products (64).

From earlier research with *L. rhamnosus* GG it is known that this strain can improve feeding tolerance and nutrient absorption (increased proliferation of villus cells) (65, 66), and could contribute to increased weight gain in children. Moreover, *L. rhamnosus* GG has shown to bind aflatoxin B1, thereby reducing its absorption in the intestine, hence reducing aflatoxin-associated pathogenicity including stunting (67–70). In view of the observations made in our study, we hypothesize that a measurable positive effect as a result of milk and probiotic yogurt consumption is likely to be found after a longer time period of consistent daily consumption of milk or yogurt. Since the present study did not include a group which did not consume a dairy product, we can only compare the impact of milk vs. probiotic yogurt.

Implications

In this study, we compared the consumption of probiotic yogurt with the consumption of plain milk in a real-life school setting. This means that besides the milk and the yogurt the children also consumed their regular maize and beans dominated diet, as well as occasional consumption of dairy products and fermented foods. Since in Southwest Uganda the majority of the schools do not serve any dairy product, and a majority of children suffer from malnutrition, we think it would be useful to conduct a study comparing not only the effects of probiotic yogurt vs. milk, but also vs. a control group that does not receive any dairy product. In such a study set-up it should be able to identify the cumulative benefits of both the nutritious components of dairy products, the bioactives produced during fermentation, as well as probiotic bacteria.

Referring to the 20,000 children that already participate in the probiotic yogurt school feeding program, we can conclude that consumption of locally produced probiotic yogurt among pre-primary school children in Southwest Uganda has gained broad acceptance. The program which started in 2018 is growing and gaining further popularity among school management committees, parents, pupils and producers in the region. It clearly provides economic opportunities through employment creation for producers, a market for milk of local farmers, and value addition to local products (24, 25). In addition, schools that participate in the program testify to have become more attractive for pupils and parents compared to schools which do not offer such nutritious and tasty dairy products to their pupils.

CONCLUSION

In this comparative interrupted times series at pre-primary schools in Southwest Uganda, we evaluated the effect of the consumption of probiotic yogurt vs. milk on the incidence of skin infections, common cold, absenteeism and anthropometric parameters among 1,116 children. Following a base-line measurement period, the pre-primary school children received probiotic yogurt or milk for a period of 8 weeks. The consumption of probiotic yogurt as conducted in this real life school feeding program showed a reducing effect on the incidence of skin infections compared to consumption of milk ($p < 0.0001$), and a reducing trend on common cold symptoms ($p = 0.09$). Anthropometric indicators and level of absenteeism did not show significant differences between yogurt and milk groups during the study period.

Since this study was done in a real-life setting, effects of confounding factors cannot be excluded. In addition, heterogeneity between control and yogurt group was observed during the 3 weeks of baseline period. Furthermore, we noticed a strong degree of cross-infection for skin infection related symptoms and common cold from the start of the study and during part of the dairy consumption period causing fluctuations in the occurrence of skin infection and common cold related incidences over time. Notwithstanding this observed positive effect and trend of probiotic yogurt on the incidences of skin infections and common cold, respectively, we consider the results of this comparative interrupted time series inconclusive. Before drawing definitive conclusions on the health impact of probiotic yogurt consumption through school feeding programs, follow-up studies are required over a longer period of time, with additional controls and a more uniform background situation for probiotic yogurt and milk groups.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Mbarara University of Science & Technology Research Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

NW, AW, AN, RK, and WS: conceptualization. NW, RK, and WS: methodology. NW, AW, and AN: investigation. NW: writing–original draft preparation. RK and WS: writing–review and editing. All authors: contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2020.574792/full#supplementary-material

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Conflict of Interest: The Yoba for Life foundation distributes and sells ready-to-use sachets with dried bacterial starter cultures at cost price, through a network of partners and volunteers to facilitate the local production of dairy and cereal-based products by controlled bacterial fermentation. African fermented products made with the Yoba starter culture, are not marketed by the foundation as such, but the Yoba for Life foundation stimulates local production and ownership, allowing income-generating activities for African small-scale entrepreneurs in the food sector. RK and WS are co-founders of the Yoba for Life foundation (2009), a non-profit organization, accredited by the Dutch Tax Authorities as a Public Benevolent Institution (PBI), which aims to promote local production and consumption of fermented products in Africa. NW is the Country Coordinator of the Yoba for Life Foundation in Uganda. In name of the Yoba for Life Foundation AN and AW provided technical support to local probiotic yogurt production units.

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Tremella fuciformis Polysaccharides Inhibited Colonic Inflammation in Dextran Sulfate Sodium-Treated Mice *via* Foxp3+ T Cells, Gut Microbiota, and Bacterial Metabolites

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Tremella fuciformis is an edible medicinal mushroom, and its polysaccharide components are found to confer various health benefits. This study identified the protective effects of polysaccharides of *Tremella fuciformis* (TPs) against dextran sulfate sodium (DSS)-induced colitis in mice. High dose of TPs (HTPs) could prevent the colon from shortening, reduce activity of colonic myeloperoxidase and serum diamine oxidase (DAO), decrease the concentration of D-lactate, and alleviate the colonic tissue damage in colitic mice. HTPs treatment stimulated Foxp3+T cells, and promoted the production of anti-inflammatory cytokines whereas it reduced the production of pro-inflammatory and the portion of immunoglobulin A (IgA)-coated bacteria, which was related to modulation of immune responses. 16S rRNA sequencing analysis showed that TPs could significantly increase gut community diversity, and restore the relative abundances of Lactobacillus, Odoribacter, Helicobacter, Ruminococcaceae, and Marinifilaceae. According to metabolomic analysis, HTPs induced specific microbial metabolites akin to that in normal mice. Tyrosine biosynthesis, tryptophan metabolism, and bile acid metabolism were influenced in the HTPs group compared with those in the DSS group. HTPs could alleviate DSS-induced colitis by immunoregulation and restored the gut microbiota and microbial metabolites. The results indicated that HTPs have potential to be developed as a food supplement to ameliorate intestinal diseases.

Keywords: colitis, gut microbiota, inflammatory responses, microbial metabolites, *Tremella fuciformis* polysaccharides

INTRODUCTION

Inflammatory bowel disease (IBD) including ulcerative colitis and Crohn's disease (CD) is a lifelong illness which poses serious threats such as chronic malabsorption of nutrients, abnormal pain, and rectal bleeding to human health. Even though the pathogenesis of IBD remains unclear, it is accepted that the interruption of the host immunity-microbiota interaction plays an essential role in the process of IBD development (1, 2). Regulatory T cells (Tregs) are critical in maintaining immune tolerance and suppressing autoimmunity, and transcription factor Foxp3 serves as a master switch for Treg (3). Immunoglobulin A (IgA) is the major effector molecule of the adaptive immunity in the gut (4). Recent research indicated that Foxp3+ T cells could repress inflammation, IgA production, and cause diversification of gut microbiota, forming a symbiotic regulatory loop (5).

Clinical symptoms of ulcerative colitis patients could be relieved to some extent with several treatments such as administration of antibiotic, but these clinical symptoms readily recur and side effects may occur, and most of available treatments have remission rates of less than 50% (6). Polysaccharides from mushroom were found to have positive effects on colonic inflammation by modifying gut microbiota in the colon, which is implicated in the maturation and education of host immune responses, protection against enteric pathogen proliferation (7, 8).

Tremella fuciformis is an edible medicinal mushroom, which has been widely cultivated. Its various bioactivities include immunomodulatory, anti-tumor, anti-oxidation, anti-aging, anti-inflammatory, repairing brain memory impairment, and lowering blood sugar and serum cholesterol levels (9, 10). The major bioactive components of *Tremella fuciformis* have been identified as polysaccharides of *Tremella fuciformis* (TPs), and under various experimental conditions different TPs fractions can be harvested, which gives TPs with a mixture of diverse polysaccharides a molecular weight ranging from 5.82×10^5 Da to 3.74×10^6 Da (10). TPs could reverse the effects of regulatory T cells on the proliferation and polarization of CD4+ T cells, down-regulate the IL-10 level, and reduce the mortality of septic mice (11). The application of TPs on IBD has not yet been explored. In this study, a new type of TPs could be isolated to ascertain the alleviation effect on DSS-induced mice and reveal the possible mechanisms related to modulation of the gut microbiome.

MATERIALS AND METHODS

Preparation of TPs

TPs were extracted with boiling water in a solid-liquid ratio of 1:20 (w/v) for 6 h. After being filtered and centrifuged, three times the volume of ethanol was added to obtain a precipitate, and the precipitate was deproteinated using the Sevage method (12). The deproteinated part was extensively dialyzed against distilled water and then applied to a 60 mm × 240 m column of DEAE-cellulose (Sigma, USA) at a flow rate of 3.0 mL/min. The column was then eluted with distilled water, 50 mM NaCl, 150 mM NaCl, and 1M NaCl at 10 mM/min, successively, and the part eluted by 50 mM NaCl was collected and lyophilized: this was defined as the TPs for further study.

Characterization of TPs

Molecular weights of TPs were determined by gel permeation chromatography (Wyatt, USA) on a Shodex OHpak SB column. The column was operated at 40°C with a flow rate of 1 mL/min. Monosaccharide composition of TPs was analyzed by liquid chromatography (Agilent 1200, USA) with a SHISEIDO C18 column. The column was operated at room temperature with a flow rate of 1 mL/min. The saccharides related to TPs were investigated by hydrolysis with dilute sulfuric acid according to the standard method of the National Renewable Energy Laboratory (CO, USA), with calibration applying a standard solution of rhamnose, arabinose, xylose, mannose, glucose, fucose, galactose, galacturonic acid, glucuronic acid, and ribose. The FT-IR spectra of TPs were collected with an FT-IR microscope (Model iN 10, Thermo Nicolet Corp.; Madison, WI, USA) equipped with a liquid nitrogen-cooled MCT detector (13).

Mice and Treatment

Thirty female C57BL/6 mice (aged 6-7 weeks, mass 17.21 ± 0.27 g) were purchased from Chengdu Dossy Experimental Animals Co., Ltd (Chengdu, China). All mice were housed in specific pathogen-free environment in a standard 12 h light-dark cycle at 25°C and had *ad libitum* access to food and water. All mice were allocated to one of four groups ($n = 6$ per group): (1) Control group (received the standard diet); (2) DSS challenge group (received the standard diet); (3) DSS challenge + 200 mg/kg TPs (LTPs group; received the standard diet plus 200 mg/kg TPs); (4) DSS challenge + 300 mg/kg TPs (HTPs group; received the standard diet plus 300 mg/kg TPs). The treatment process lasted for two weeks. Mice were orally treated with TPs or normal saline (Control and DSS groups) once daily for 14 days, starting 7 days before the induction of colitis. Acute colitis was induced by 3.5% DSS given in distilled water for days 8-14 except to those in the control group. At day15, mice were sacrificed to yield samples for subsequent experiments.

Serum Biochemical Analysis

Blood samples were collected from the coeliac artery, and then centrifuged at 2000*g* for 15 min to harvest serum for analysis. The D-lactate concentration and diamine oxidase (DAO) activity in serum were determined with commercial assay kits H263 and A088-1 from Nanjing Jiancheng Bioengineering Institute (Nanjing, China), respectively.

Bodyweight, Colon Length, Myeloperoxidase Activity, and Histological Analysis

After anesthesia the bodyweight of mice was recorded. Once the mice were sacrificed, the length of the colon was measured, and

samples were processed for the following analysis. Colonic samples were homogenized in phosphate-buffered saline (PBS) buffer (pH 7.4) and centrifuged at 14,000*g* for 15 min at 4°C, and the supernatant was used for MPO activity tested with myeloperoxidase assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The colon tissue specimens were fixed with 10% neutral buffered formalin at room temperature, dehydrated in a graded ethanol series, and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (H & E) to identify histological change. Images were acquired using a microscope (Leica Microsystems, Wetzlar, Germany), and the villus height and crypt depth were measured according to the stained images with software Image-Pro Plus 6.0. The histological analysis was performed by an independent researcher in a single-blind manner (14).

Determinations of Intestinal Cytokine and Nuclear Receptor PPAR-γ Concentrations

After mice were sacrificed, colonic tissue was collected, weighed, and immediately preserved with liquid nitrogen. We thawed the specimens before testing and kept them at 2 to 8°C. We homogenized the specimens on ice with normal saline in the ratio of 1:9 (w/v) using a homogenizer. The homogenates were centrifuged at 3000*g* for 15 min at 4 °C, and the supernatant was harvested for subsequent analysis. The concentrations of different cytokines (IFN-γ, IL-1β, IL-6, TGF-β, IL-10, and TNF-α) in the colonic tissue supernatant were quantified using enzyme-linked immunosorbent assay (ELISA) kits (Shanghai ZCI Bioscience Co., Ltd, Shanghai, China) according to the manufacturer's instructions. The PPAR-γ concentration was determined with a ZC-38225 Mouse PPAR-γ ELISA Kit (Shanghai Zhuo Cai Technology Co., Ltd, Shanghai, China) referring to the manufacturer's instructions.

Quantification of Immune Cells in the Colonic Mucosa and IgA-Coated Bacteria in Intestinal Contents

Flow cytometric analysis of IgA-coated bacteria was implemented as described elsewhere (5) with some modifications. Intestinal contents were placed in a 1.5 mL tube and washed with 200 μL PBS. The supernatant was collected and passed through cell strainer, and then centrifuged at 500*g* for 5 min. The supernatant was discarded, and the precipitate was resuspended with 100 μL PBS for later use. FITC labeled anti-mouse IgA (C10-1) from BD Biosciences (5μL) was added to each tube containing the intestinal content specimens. These samples were protected from light at 4°C for 30 min, washed twice with 1 mL PBS, centrifuged at 500*g* for 5 min, the supernatant was discarded, and the cells were resuspended with 500 μL PBS. These samples were analyzed by flow cytometry.

Lymph node cells were collected from mesentery and analyzed for Foxp3+ Tregs, which was conducted with reference to Pei et al. (15). Lymph node cells were first stained with the following antibodies (BioLegend, CA): FITC-conjugated mAb to CD4 (RM4-5) and Alexa Fluor® 647-conjugated mAb to CD25 (PC61). After fixation, intracellular staining with antibody of PE-conjugated mAb to Foxp3 (150D) was performed (BioLegend, CA). Data were acquired on a flow cytometry analysis (CytoFLEX, Beckman Coulter, USA). Except for this group, the lymph node samples were divided into another four groups, including a Blank group (unstained), CD4 single staining group, CD25 single staining group, and a Foxp3 single staining group.

Gut Microbiota Analysis

Samples of intestinal contents from control group, DSS challenge group, and HTPs group were collected. Total genome DNA from intestinal content was extracted by using the CTAB/SDS method and was subjected to 16S amplifications using primers designed to incorporate both the Illumina adapters and a sample barcode sequence, allowing directional sequencing that covers variable region V4 (Primers: 515 F [GTGCCAGCMGCCGCGGTAA] and 806R [GGACTACHVGGGTWTCTAAT]. PCR reactions were performed with Phusion® High-Fidelity PCR Master Mix (New England Biolabs, USA). Sequencing libraries were generated using Ion Plus Fragment Library Kit 48 rxns (Thermo Scientific, USA) following manufacturer's recommendations. The library was sequenced on an Ion S5™ XL platform and 400 bp/600 bp single-end reads were generated. All the results were based on sequenced reads and operational taxonomic units (OTUs). Analysis of 16S sequences was undertaken by UPARSE software (v7.0.1001), and sequences with ≥ 97% similarity were assigned to the same OTUs. The Silva Database (https://www.arb-silva.de/) was used based on the Mothur algorithm to annotate taxonomic information. Alpha diversity including observed-species, Chao1, Shannon, Simpson, ACE, good coverage, and beta diversity were both calculated with QIIME (Version1.7.0) and displayed with R software (Version 2.15.3). All 16S rRNA sequencing data were saved in the National Center for Biotechnology Information and can be accessed in the Short Read Archive under accession number PRJNA679459 (http://www.ncbi.nlm.nih.gov/bioproject/679459).

Measurement of Fecal Metabolomics

Samples of intestinal contents of control group, DSS challenge group and HTPs group specimens were collected. Each sample was individually ground with liquid nitrogen and the homogenate was resuspended with pre-chilled 80% methanol and 0.1% formic acid. The samples were centrifuged, and supernatant was diluted to final concentration containing 60% methanol by LC-MS grade water. The samples were subsequently transferred to a fresh Eppendorf tube with 0.22-μm filter, then centrifuged. Finally, the filtrate was injected into the LC-MS/MS system for analysis.

LC-MS/MS analyses were conducted using a Vanquish UHPLC system (Thermo Fisher) coupled with an Orbitrap Q Exactive™ series mass spectrometer (Thermo Fisher). Samples were injected onto an Hypersil Gold column (100 mm × 2.1 mm, 1.9 μm) using a 16-min linear gradient at a flow rate of 0.2mL/min. The eluents for the positive polarity mode were eluent A (0.1% FA in water) and eluent B (methanol). The eluents for the negative polarity mode were eluent A (5 mM ammonium acetate,

pH 9.0) and eluent B (Methanol). The solvent gradient was set as follows: 2% B, 1.5 min; 2-100% B, 12.0 min; 100% B, 14.0 min; 100-2% B, 14.1 min; 2% B, 16 min. Q Exactive mass series spectrometer was operated in positive/negative polarity mode with spray voltage of 3.2 kV, capillary temperature of 320°C, sheath gas flow rate of 35 arb and auxiliary gas flow rate of 10 arb. The metabolites were annotated using the KEGG database (http://www.genome.jp/kegg/), HMDB database(http://www.hmdb.ca/) and LIPID MAPS® Structure Database (LMSD) (http://www.lipidmaps.org/). Principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed at MetaX. Univariate analysis (*t*-test) was conducted to determine the statistical significance (*P*-value). Those metabolites with VIP > 1, *P*-value < 0.05, and fold-change ≥ 2 or fold-change ≤ 0.5 were considered differential metabolites.

Data Analysis

Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Tukey test using SPSS 22.0 software (IBM, Chicago, IL, USA). Data are presented as mean ± standard deviation (SD) or as a scatter plot. The correlations between relative abundances of OTUs and microbial metabolites were analyzed by Spearman's correlation (SPSS 22, IBM): $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Molecular Weight, Monosaccharide Composition, and Infrared Spectral Analysis of TPs

Molecular weights of TPs harvested from HP-GPC detection are listed in **Table 1**. M_w and M_n values of TPs were 288.6 and 268.8 kDa, respectively. The *D* value (M_w/M_n) was 1.074, suggesting that the TPs were relatively pure. Molecular weights of TPs were mainly distributed between 246 and 305 kDa (**Table 2**). The monosaccharide components of TPs are displayed in **Table 3**. The main monosaccharide sector comprised mannose followed by glucuronic acid, xylose, fucose, glucose, galactose, arabinose, rhamnose, and ribose.

Infrared spectroscopy reflected the characteristic absorption peak of polysaccharides used when analyzing the structure of these TPs. **Figure 1** shows that absorption peaks at 3300-3000 cm^{-1}, 1675-1640 cm^{-1}, and 1000-675 cm^{-1} indicated the presence of double bonds. The presence of a formyl group was evinced by the absorption peaks at 1300-1000 cm^{-1} and 769-659 cm^{-1}. The absorption peaks at 3000 cm^{-1} and 1600 cm^{-1} suggested the presence of an aromatic ring. Strong absorption of 3500 to 3200 cm^{-1} and 1300 to 1000 cm^{-1} showed the presence of an alcohol hydroxyl group. According to infrared spectroscopy analysis, there was 78.24% similarity between TPs and hemicellulose.

TPs Ameliorated Clinical Symptoms in the DSS-Induced Colitis Mice Model

The typical symptoms and pathological changes of colitis involve bodyweight loss, shortening of colon, serum DAO activity, D-lactate concentration increase, hyperplasia of crypts, *etc.* (16–18). As shown in **Figures 2A, B**, a significant decrease of bodyweight and shortening of colon could be found after DSS treatment. TPs improved weight and length, with HTPs being the most beneficial. Intestinal epithelial barrier deficiencies along with abnormal intestinal permeability are crucial pathogenic factors in IBD (19). In our study, HTPs treatment reduce DAO activity and D-lactate concentration in serum which reflects the intestinal barrier integrity compared with DSS group (**Figure 2**). Villus height, crypt depth, and villus length to crypt depth ratio (VCR) reflect gross intestinal morphology (20): as shown in **Figure 3**, HTPs treatment prevented loss of villus height and VCR of colon, and largely ameliorated colon inflammatory symptoms, including relative intact surface epithelium, less inflammatory cell infiltration, and mild submucosal oedema. Thus, TPs treatments significantly reduced the abnormal histopathological changes in the colonic tissues. These results indicated that pre-treatment with TPs could effectively inhibit the severity of colitis in DSS-treated mice.

TABLE 1 | Molecular weights.

Mw/Mn	Mz/Mn	Mn, kDa	Mp, kDa	Mw, kDa	Mz, kDa
1.074	1.177	268.8	309.0	286.6	316.4

TABLE 2 | Molecular weight distribution.

Molecular weight distribution	154000.0-246000.0 g/mol	246000.0-305000.0 g/mol	305000.0-400000.0 g/mol	400000.0-770484.0 g/mol
%	15.2	42.0	36.0	6.8

TABLE 3 | Monosaccharide composition.

Monosaccharide composition (mg/kg)								
Mannose	Ribose	Rhamnose	Glucuronic acid	Glucose	Galactose	Xylose	Arabinose	Fucose
91120.57	337.73	623.67	29067.09	2086.89	1656.21	25035.97	1540.07	21388.01

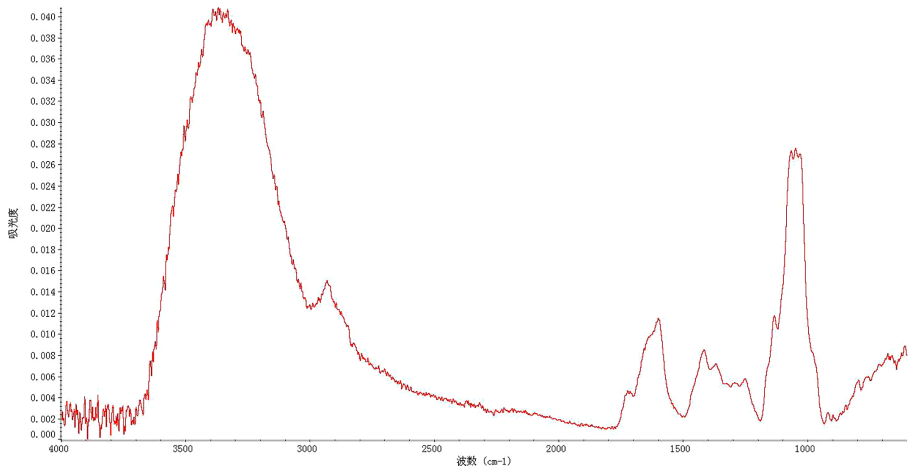

FIGURE 1 | FT-IR spectrum of TPs.

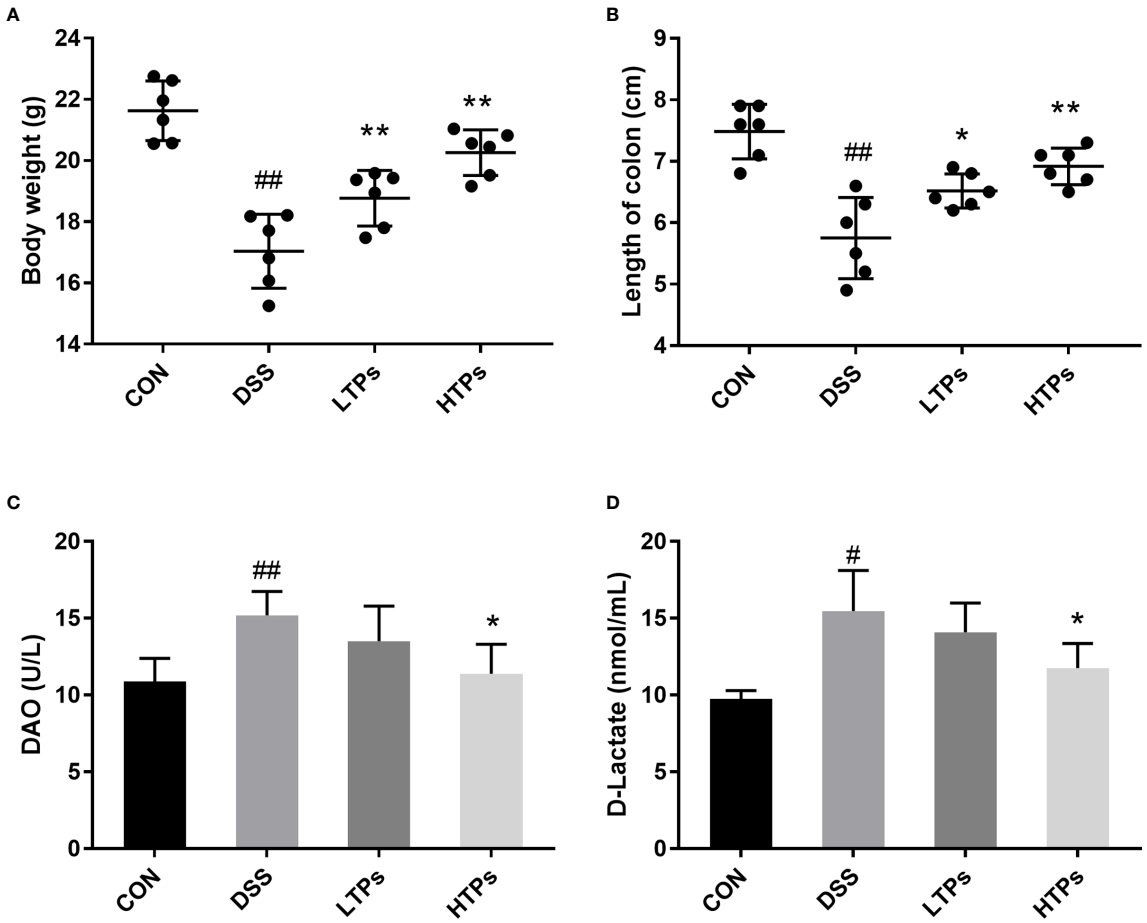

FIGURE 2 | TPs ameliorated DSS-induced clinical symptoms in mice. **(A)** Bodyweights of mice were measured after the experiment. **(B)** Length of colon. **(C)** DAO activity in serum. **(D)** D-lactate concentration in serum. CON, Control group; DSS, DSS challenge group; LTPs, DSS challenge + 200 mg/kg TPs; HTPs, DSS challenge +300 mg/kg TPs. All values are represented as mean ± SD (n = 6). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *v*. normal group. $^{*}p < 0.05$, $^{**}p < 0.01$ *v*. control group.

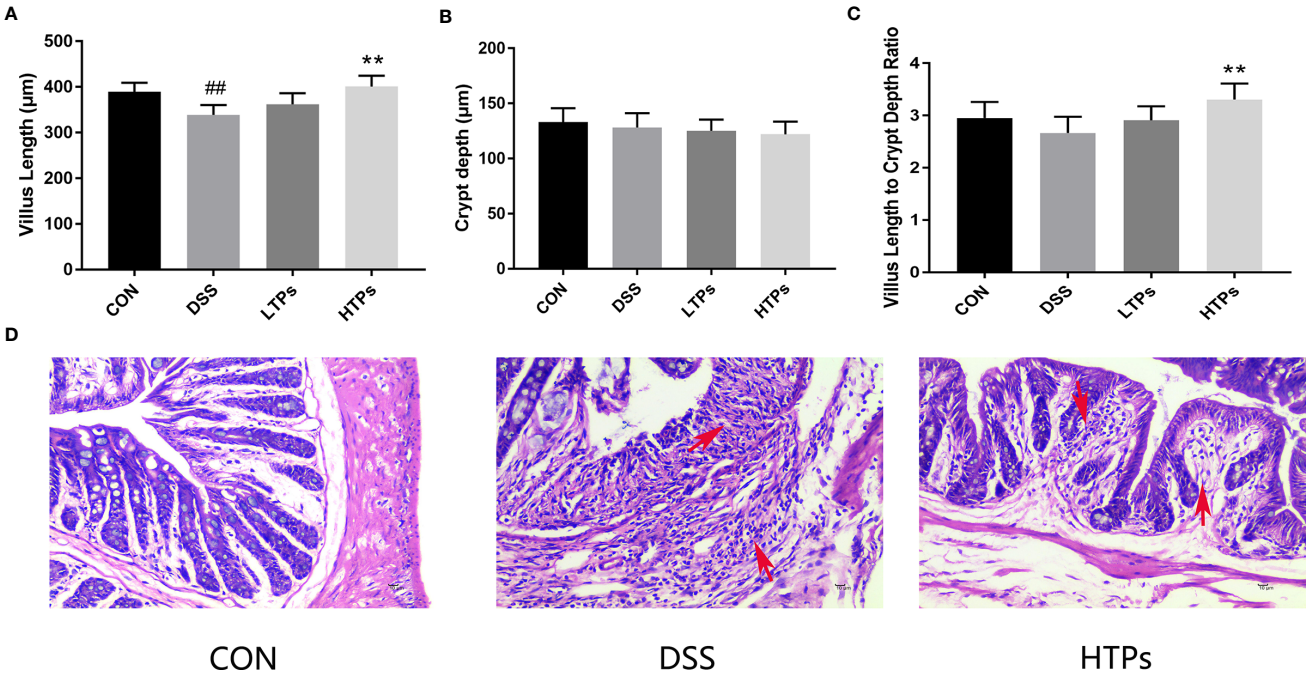

FIGURE 3 | Effects of HTPs on colonic morphology in DSS-challenged mice. **(A)** Villus height. **(B)** Crypt depth. **(C)** Villus length to crypt depth ratio (VCR). **(D)** Representative images of H & E staining of the colon tissue (magnification 400×). CON, control group; DSS, DSS challenge group; LTPs, DSS challenge + 200 mg/kg TPs; HTPs, DSS challenge +300 mg/kg TPs. All values are represented as mean ± SD (n = 6). $^{\#\#}p < 0.01$ *v*. normal group. $^{**}p < 0.01$ *v*. control group.

Effects of TPs on Inflammation in DSS-Induced Colitis Mice

Colitis is associated with chronic inflammation resulting from loss of IL-10 and increase of pro-inflammatory cytokines such as IFN-γ, IL-1β, IL-6, TGF-β, and TNF-α (21, 22). PPAR-γ is a member of a superfamily of nuclear receptors, which possess crucial anti-inflammatory activity, and can inhibit the expression of IL-1β and TNF-α (23). The values of pro-inflammatory cytokines were remarkably increased in the DSS group while anti-inflammatory cytokines were notably decreased compared with those in the control group (**Figure 4**). Treatment with TPs significantly enhanced anti-inflammatory cytokines IL-10 and lowered pro-inflammatory cytokines (IFN-γ, IL-1β, IL-6, TGF-β, and TNF-α). Additionally, the HTPs had better anti-inflammatory effect. Both low-dose and high-dose TPs treatment notably elevated PPAR-γ concentration in colonic tissue. It has been shown that promoting PPAR-γ activation could protect mice from DSS-induced colitis (24). This result offered further proof of the effects of TPs in alleviating DSS-induced colitis. The activity of MPO was an index of neutrophil infiltration and inflammation, and the level of MPO activity reflected the level of colonic inflammation (25). MPO level in DSS-treated colon tissue was found to increase significantly (**Figure 4H**). The HTPs group decreased the MPO level, indicating that HTPs (300 mg/kg) alleviated inflammation in the colon.

It has been shown that Foxp3+T cells regulated the expansion of naïve CD4+T cells and their production of pro-inflammatory cytokines. The production of pro-inflammatory cytokines such as TNF-α and IFN-γ decreased while the level of anti-inflammatory cytokine IL-10 increased in the presence of Foxp3+T cells (26). By flow cytometry analysis, the proportion of Treg cells (CD4+CD25+FoxP3+) in the mesenteric lymph nodes of the HTPs-treated group was significantly higher than in the DSS group (**Figures 5A, B**), which shows that HTPs treatment promoted Foxp3+T cells. IgAs are mostly produced in mucus membranes. Leakage of serum into the gut at sites of inflammation could yield a higher percentage of IgA-coated bacteria in IBD patients (27, 28). In our study, the DSS treatment increased IgA-coated bacteria proportion in the gut as shown in **Figures 5C, D**; however, feeding with HTPs notably decreased the proportional increase therein. IgA played an important role in the balance of gut bacterial communities (5, 27). HTPs treatment affected the expansion of Foxp3+T cells and IgA production to reduce the gut inflammatory status and adjusted the balance of the gut microbiome.

Gut Microbiota Analysis

Perturbations in the intestinal microbiome induced host physiology altering by affecting homeostasis, barrier function, innate and adaptive immune responses, and metabolism (29, 30). Illumina Miseq (16S rRNA gene) was employed to

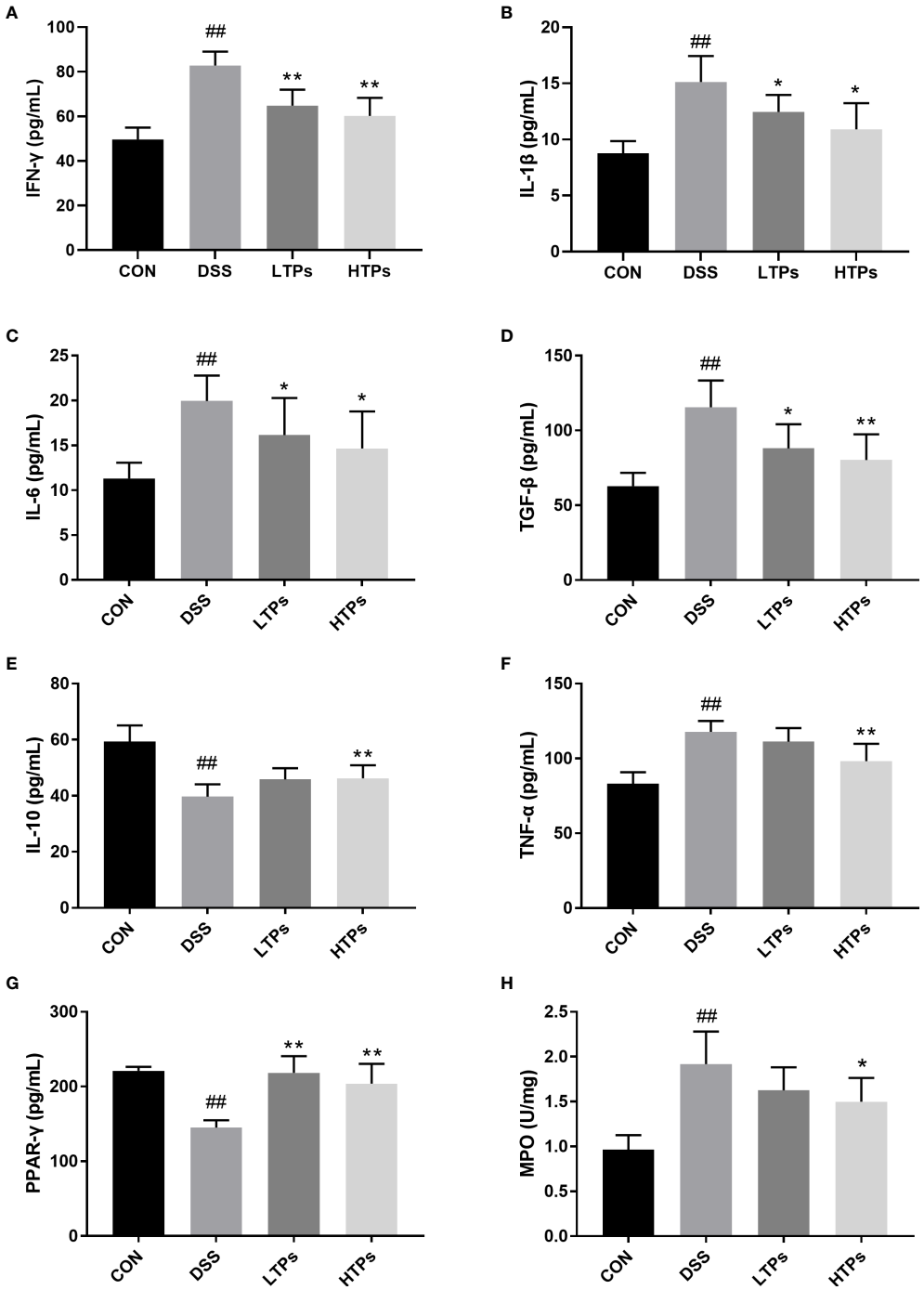

FIGURE 4 | Quantification of the concentrations of IFN-γ **(A)**, IL-1β **(B)**, IL-6 **(C)**, TGF-β **(D)**, IL-10 **(E)**, TNF-α **(F)**, PPAR-γ **(G)** and MPO **(H)** activity in colon. All values are represented as mean ± SD ($n = 6$). CON, Control group; DSS, DSS challenge group; LTPs, DSS challenge + 200 mg/kg TPs; HTPs, DSS challenge +300 mg/kg TPs. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *v*. normal group. $^{*}p < 0.05$, $^{**}p < 0.01$ *v*. control group.

characterize the overall pattern of gut microbiota community in control, DSS- and HTPs-treated mice. In our study, alpha and beta diversity indices were analyzed to illuminate bacterial richness, diversity, and structural differences in each group (**Table 4**, **Figure 6A**). According to Shannon and Simpson indices, DSS treatment decreased gut community diversity to a significant extent. With HTPs treatment, the Simpson indices increased again. Referring to Chao 1 and Ace indices, DSS treatment induced no significant change, but HTPs treatment decreased community richness compared with the specimens in the DSS group. Analysis of

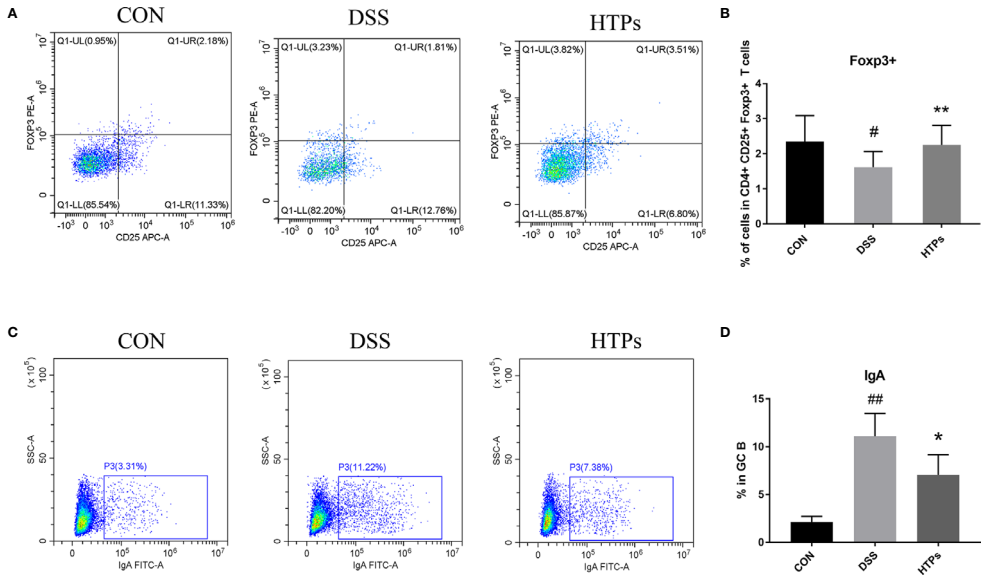

FIGURE 5 | Flow cytometry analysis of the populations of Treg (CD4+CD25+Foxp3+) and IgAs from C57BL/6 mice. Representative dot plots from mesenteric lymph nodes and intestinal contents. Treg (CD4+CD25+Foxp3+) and IgAs in **(A, C)** respectively. Relative expression of Treg (CD4+CD25+Foxp3+) and IgAs in **(B, D)**. CON, Control group; DSS, DSS challenge group; LTPs, DSS challenge + 200 mg/kg TPs; HTPs, DSS challenge +300 mg/kg TPs. All values are represented as mean ± SD (n = 6). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *v*. normal group. $^{*}p < 0.05$, $^{**}p < 0.01$ *v*. control group.

beta diversity with PCoA (**Figure 6A**) showed that gut microbiota in control, DSS and HTPs groups were significantly different in terms of the cumulative contributions made by the first two principal components which accounted for 96.72% of all species herein. As a result, DSS and HTPs treatment caused significant changes in the composition of gut microbiome.

Figure 6B illustrates the microbial composition at phylum level in HTPs, control, and DSS groups. Firmicutes and Bacteroidetes are important in adjusting absorption, energy transformation, and glucose metabolism which are parts of the body's energy balance mechanism (31). The higher ratio of Firmicutes/Bacteroidetes in the HTPs group may have the potential ability to enhance the capacity to absorb calories from foods by gut microbes compared with control and DSS groups. HTPs treatment could notably alter the abundances of Lactobacillus, Odoribacter, Helicobacter, Ruminococcaceae, Lactobacillaceae, and Marinifilaceae to normal level compared with the control group (**Figure 7**). The abundances of Lactobacillaceae and Lactobacillus were significantly elevated after HTPs treatment compared with DSS treatment, which is consistent with Yanhui Han's finding that strawberry restored Lactobacillus in colonic mice and Lactobacillus possessed protective immunoregulatory properties against colonic inflammation (32, 33). Lactobacillaceae generated formic acid, lactic acid, acetic acid, and ethanol by fermentation of glucose and pectin, and acetic acid was associated with improved energy and lipid metabolism (34). The significantly decreased abundance of Helicobacter in HTPs group mice compared with control group helped to restore healthy gut microbiome as infection with Helicobacter spp. has been reported to cause IBD in immunodeficient mice (35). Ruminococcaceae in the individuals who were suffering from chronic inflammatory bowel disease were found to be elevated, but HTPs helped to lower the abundance of Ruminococcaceae in the colon (36). Our results pertaining to Odoribacter conflict with earlier findings suggesting that Odoribacter is more abundant in healthy individuals, and the decrease of Odoribacter is related to host inflammation (37). Odoribacter is a producer of acetic acid, propionic acid, and butyric acid, and the reduction of Odoribacter resulted in a shortage of short-chain fatty acids (SCFA), which led to host intestinal inflammation (38); however, the analysis suggests a correlation between the microbiome and metabolites in our study (**Figure 9**), and Odoribacter has no significant relationship with SCFA. As the gut microbiome is diverse, Odoribacter occupies a small part thereof, which may contribute little in the present study: much remains to be explored with regard to this issue.

Cladogram and linear discriminant analysis (LDA) (**Figures 6C, D**) were used to demonstrate the hierarchy and abundance of

TABLE 4 | Alpha diversity parameters assessed by Ace, Chao1, Shannon, and Simpson indices.

Item	Treatment		
	CON	DSS	HTPs
Shannon index	6.27 ± 0.07	5.71 ± 0.20**	5.72 ± 0.08
Simpson index	0.97 ± 0.00	0.94 ± 0.01**	0.96 ± 0.00##
Chao1 index	393.09 ± 30.64	371.89 ± 24.59	323.8794 ± 12.75#
ACE index	390.55 ± 23.95	374.08 ± 24.29	328.50 ± 13.87#

*CON, Control group; DSS, DSS challenge group; HTPs, DSS challenge 300 mg/kg TPs. **p < 0.01 v. the CON group. #p < 0.05 or ##p < 0.01 v. the DSS group.*

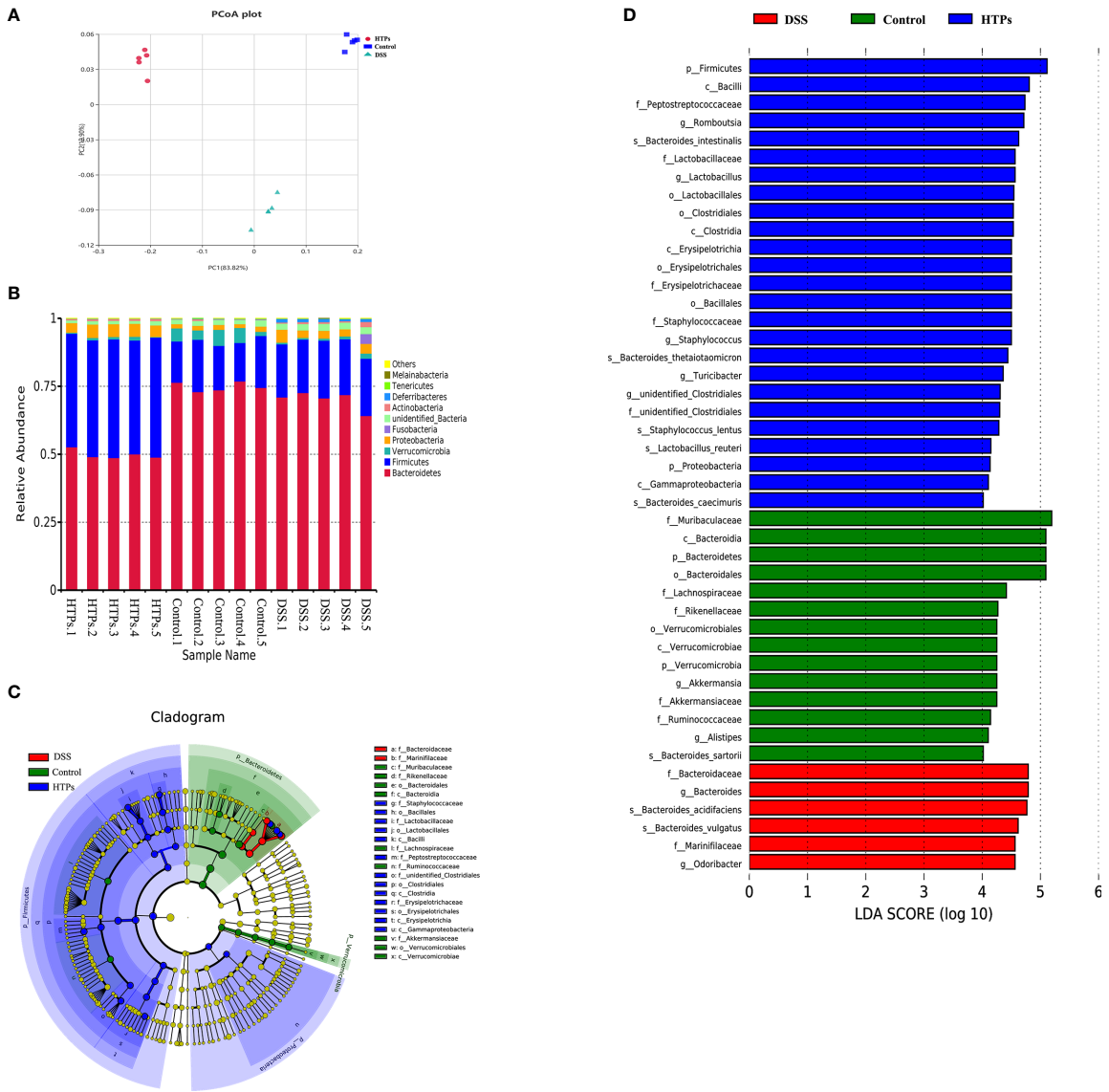

FIGURE 6 | **(A)** Beta diversity parameters measured with PCoA plot. Each point designates an individual sample, and the points of different colors represent various treatments. The distance between different points demonstrates the similarity or differences of the microbial community structure. **(B)** Microbial composition at phylum levels in HTPs, control and DSS groups. Linear discriminant analysis effect size (LEfSe) analysis of microbiota in HTPs, control and DSS groups: **(C)** taxonomic cladogram demonstrating highly abundant taxa across various treatments. **(D)** taxa that meet an LDA score threshold of > 4 (n = 5).

gut microbiome between different groups. Firmicutes, Proteobacteria, Verrucomicrobia, and Bacteroidetes were the dominant phyla in all groups. LDA also showed the highest abundance of Firmicutes in the HTPs group which was in agreement with previous microbial composition analysis. The higher abundance of Bacteroides in DSS group in this study was consistent with reports that commensal Bacteroides play a potential role in proinflammation in colitic mice (39). Lactobacillus also exhibited a higher LDA score in the HTPs group. Generally, HTPs treatment modulated the perturbed gut flora in colitic mice to various degrees by increasing the abundances of Lactobacillus and Lactobacillaceae, and reducing the abundances of Odoribacter, Helicobacter, Ruminococcaceae, and Marinifilaceae.

Metabolite Analysis

The gut microbiota can offer energy and vitamins for the host by microbial metabolites and directly influence a variety of aspects of metazoan physiology (40). Microbial metabolites are crucial for host-microbial interactions and regulate host immune responses. LC-MS/MS was conducted to analyze the colonic digesta metabolic profiles: 3491 metabolites were detected and

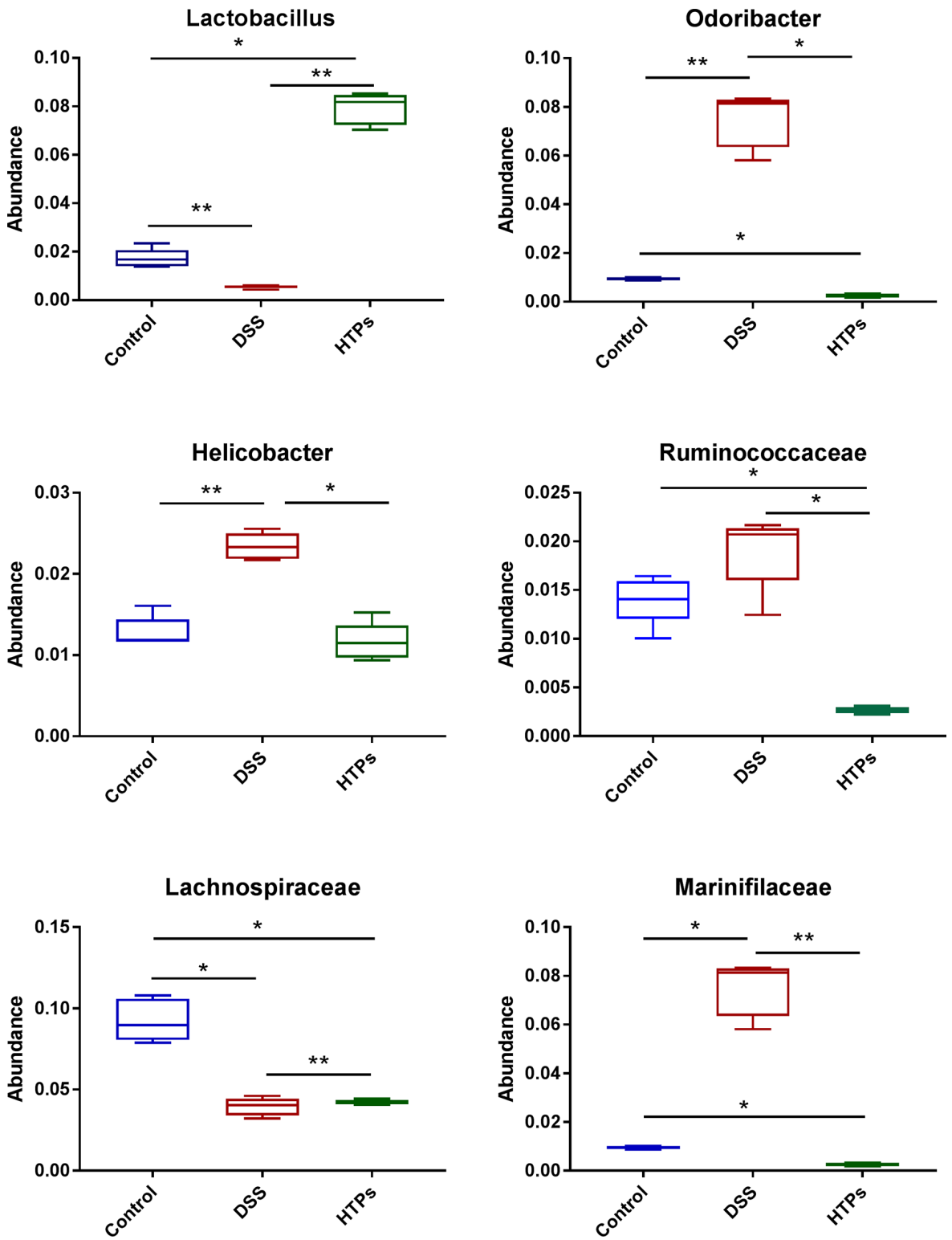

FIGURE 7 | Relative abundances of Lactobacillus, Odoribacter, Helicobacter, Ruminococcaceae, Lactobacillaceae, and Marinifilaceae across each grouped microbiome. All values are represented as mean ± SD (n = 5).*p < 0.05, **p < 0.01.

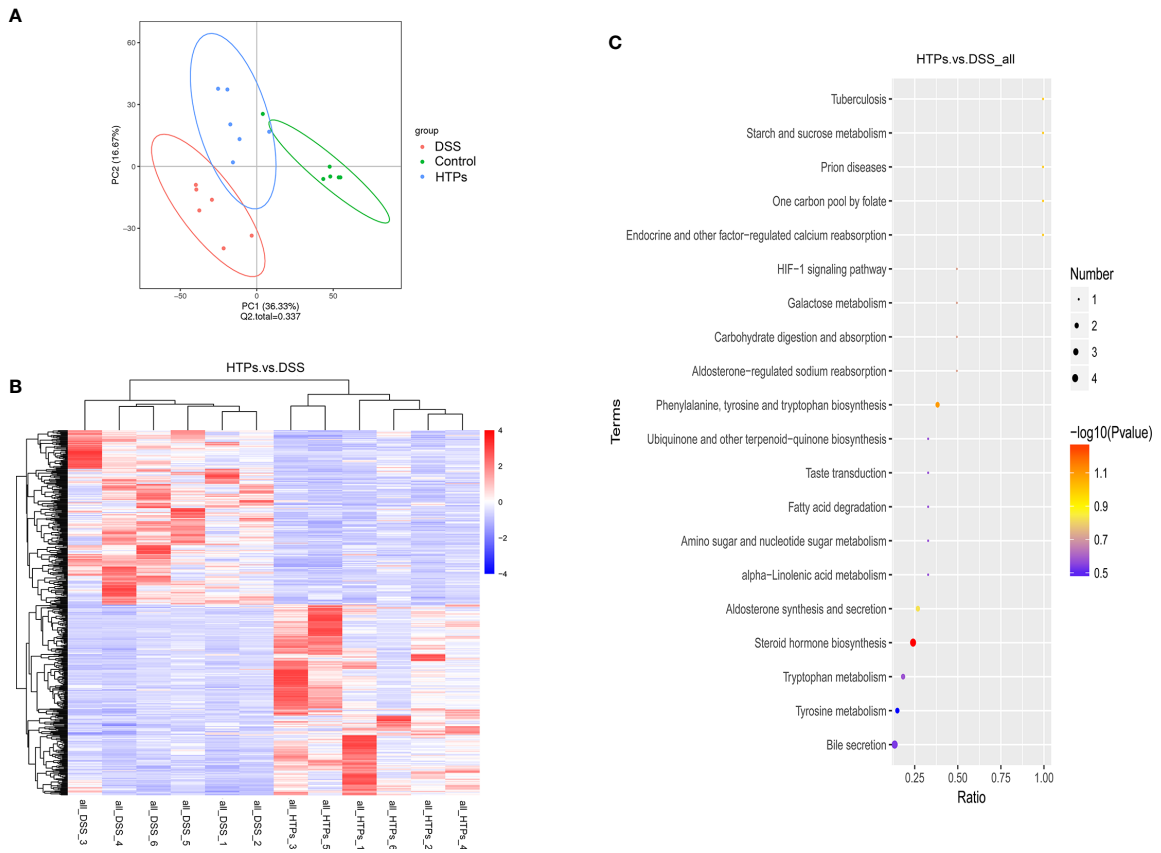

FIGURE 8 | **(A)** Partial least squares discriminant analysis (PLS-DA) score plots comparing total fecal metabolites in HTPs, DSS and control groups. **(B)** Hierarchical clustering significantly changed metabolites of HTPs and control groups. VIP > 1.0, FC > 1.5, or FC < 0.667 and *P*-value < 0.05. **(C)** KEGG pathway enrichment of the significantly changed metabolites of HTPs and control groups. The *x*-coordinate is the ratio of the number of significantly changed metabolites in the corresponding metabolic pathways to the total number of metabolites in the identified pathway. The higher the value, the greater the enrichment of significantly changed metabolites in the pathway. The color of the dot represents the *P*-value of the hypergeometric test: the smaller the *P*-value, the more reliable and statistically significant the test. The size of the dot represents the number of significantly changed metabolites in the corresponding pathway. The bigger the dot, the greater the number of significantly changed metabolites in the pathway ($n = 5$).

454 of them were significantly changed because of HTPs exposure (**Supplementary Table 1**). Partial least squares discriminant analysis (PLS-DA) score plots showed significant changes in the composition of fecal metabolites of different groups. In this study, fecal metabolites in HTPs, control, and DSS groups were almost separated from each other in terms of their different compositions (**Figure 8A**). Clustering based on different metabolites implied that all samples were independent and unique in the metabolome dimensions, and 217 significantly changed metabolites were down-regulated and 237 were up-regulated (**Figure 8B**). Kyoto Encyclopedia of Genes and Genomes (KEGG) was adopted to analyze the top 20 enriched pathways of significantly different metabolic changes between DSS and HTPs groups (**Figure 8C**). The metabolic pathways of bile secretion, phenylalanine, tyrosine, and tryptophan biosynthesis, tyrosine metabolism, tryptophan metabolism, and steroid hormone biosynthesis, *etc.* were enriched. A significant elevation of L-tyrosine (Com_156_pos) was observed in HTPs group, and this increasement also occurred in fermentation supernatants of fecal inocula from healthy volunteers and edible mushroom *Pleurotus eryngii, Pleurotus ostreatus* and *Cyclocybe cylindracea* (*Basidiomycota*), which protected human colon adenocarcinoma Caco-2 against tert-butyl hydroperoxide (41). Referring to correlation analysis of significantly changed metabolites and microbiomes, L-tyrosine was elevated with the increased count of Lactobacillus and decreased counts of Odoribacter, Helicobacter, and Ruminococcaceae (**Figure 9**). We wonder whether HTPs may increase L-tyrosine levels through restoring Lactobacillus, Odoribacter, and Helicobacter. The reduction of intestinal tryptophan catabolites may have effects on the severity of IBD (42). The tryptophan metabolites of HTPs group, Xanthurenic acid (Com_5405_pos), and Kynurenic acid (Com_763_pos) were up-regulated compared with those in the DSS group (**Figure 10**). Thus, HTPs may protect the colon from inflammation through elevating the level of tryptophan catabolites. Due to indole derivatives being an

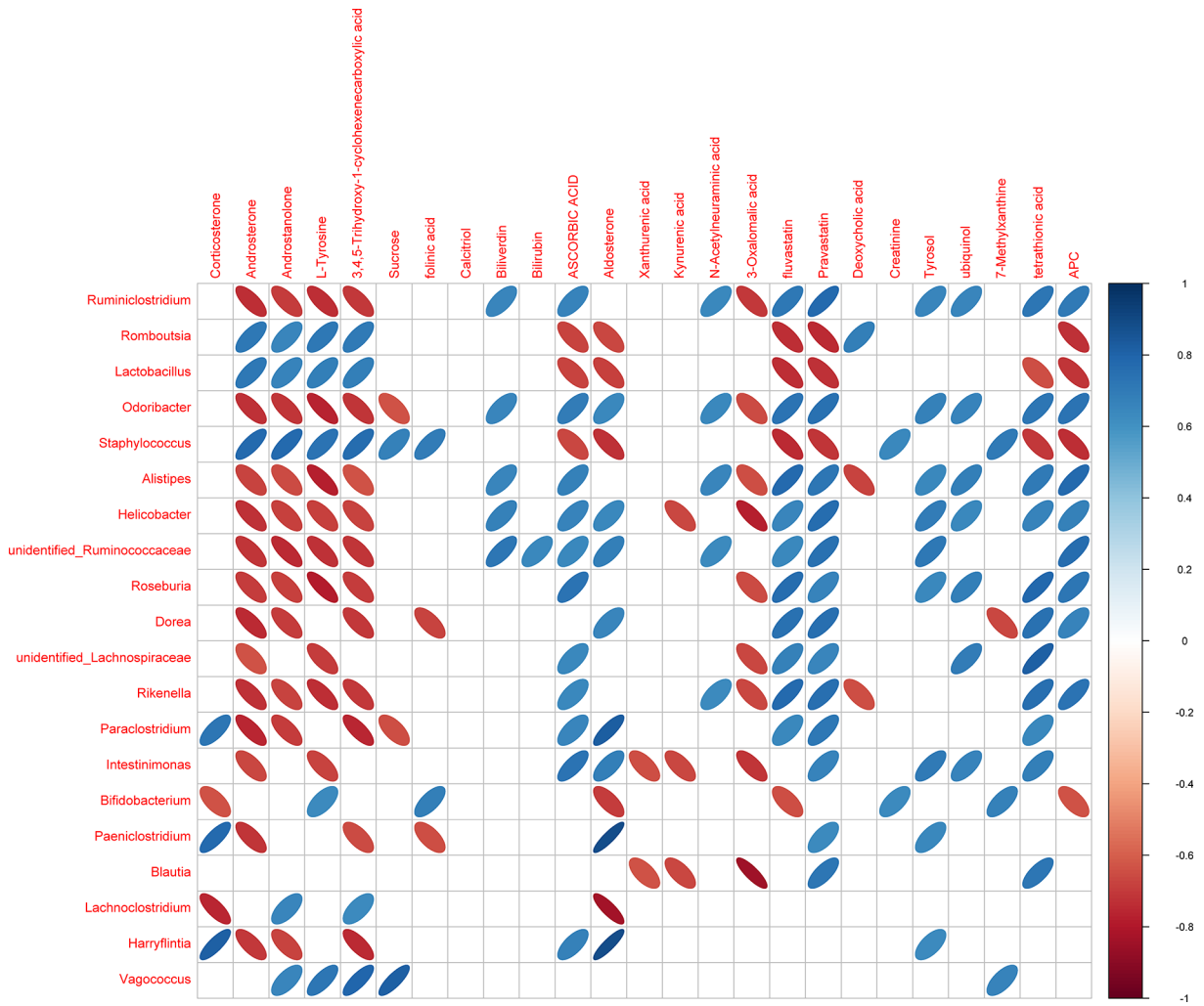

FIGURE 9 | Heatmap of Spearman's correlation between the microbiome and metabolites. The abscissa denotes the top-20 different metabolites and the ordinate is the top-10 different microbiomes at generic level in the HTPs and DSS groups. The color represents a significant correlation ($P \leq 0.05$), and the color scale denotes Spearman's correlation from blue (positive correlation) to red (negative correlation) ($n = 5$).

indispensable part of the tryptophan catabolites generated by the gut microbiota, they affect host physiology in numerous ways, such as decreasing intestinal permeability, altering innate and adaptive immune responses, suppressing appetite, and inducing anti-oxidative and anti-inflammatory effects (43). In our study, indole derivatives detected in HTPs fecal matter including 5-methoxy-3-indoleaceate (Com_9624_pos), 5-hydroxyindole (Com_4464_pos), methyl (3-hydroxy-2-oxo-2,3-dihydro-1H-indol-3-yl) acetate (Com_12338_pos), 5-hydroxyindole-3-acetic acid (Com_317_pos), and 5-hydroxyindoleacetic acid (Com_972_neg), and these were all significantly increased compared with levels found in the DSS group. It is found that *Lactobacillus* strains metabolizing tryptophan could attenuate intestinal inflammation *via* aryl hydrocarbon receptor activation, especially, *Lactobacillus reuteri* which actives the aryl hydrocarbon receptor by way of indole derivatives to regulate intraepithelial CD4+ T helper cells in their transformation to immunoregulatory T cells (CD4+ CD8αα double-positive intraepithelial lymphocytes). In our study, the prominent increase in *Lactobacillus* in the HTPs group, whether it induced Foxp3+ T cell expansion by elevating tryptophan metabolites, required further proof. Bile acids were metabolized in the intestine by the gut microbiota, and the deconjugated primary bile acids were metabolized through gut microbial 7-dehydroxylation into secondary bile acids (lithocholic acid and deoxycholic acid) in the colon (44). In our study, the secondary bile acid (deoxycholic acid, Com_32_neg) was detected at significantly increased level compared with DSS group specimens in microbial metabolites. The analysis of the correlation between the microbiome and metabolites (**Figure 9**) indicated that deoxycholic acid was positively correlated with *Romboutsia*, which belongs to the

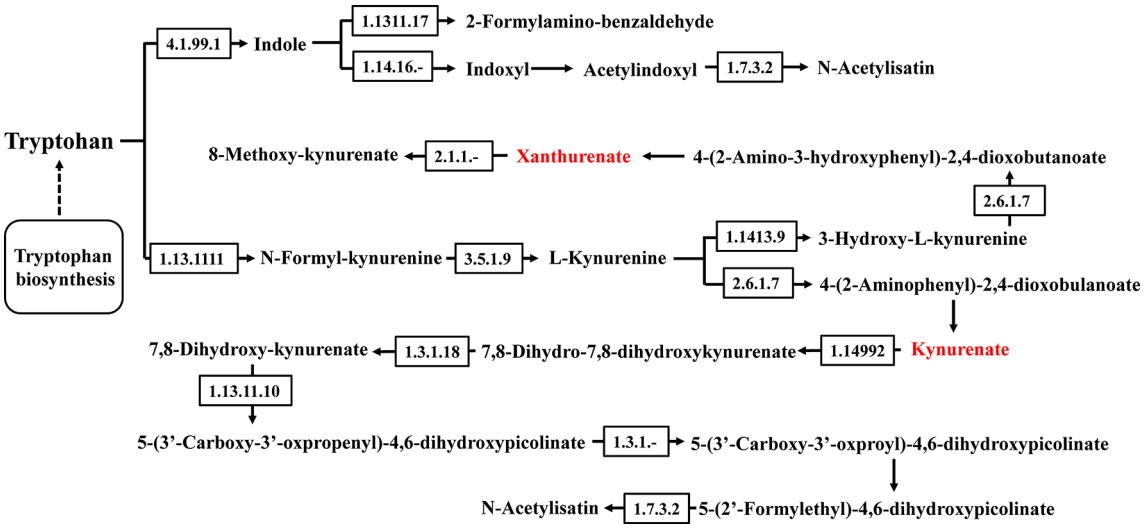

FIGURE 10 | Tryptophan metabolism pathway.

Clostridium species. It is found that bacteria with capability to produce secondary bile acids were identified in Clostridium as belonging to the Firmicutes phylum (45), showing that Romboutsia may stimulate deoxycholic acid generation in the intestine to modify bile acid metabolism. Thus, HTPs may adjust microbial metabolites in colitic mice to a normal level through influencing tyrosine biosynthesis, tryptophan metabolism, and bile acid metabolism.

CONCLUSION

HTPs treatment exerted a profound influence on colitis in mice by targeting host factors and microorganisms, including microbial physiology and metabolites. With HTPs treatment the typical symptoms and pathological changes of colitis were in remission. This effect was closely associated with immunoregulation of the host which as evinced by the fact that Foxp3+ T cell expansion and IgA-coated bacteria reduction, augment anti-inflammatory cytokines and decrease pro-inflammatory cytokines in colitic mice. HTPs treatment led to gut flora compositional changes and modulated microbial metabolites in HTPs group in a similar manner to that in the control group. Results of gut microbiota and microbiota metabolism analyses showed that HTPs improved the metabolites and metabolic pathways related to the change in inflammation caused by colitis and had an effective influence on intervention in DSS-induced colitis. This is the first study to ascertain the underlying mechanism of TPs in colitis through multi-path analysis of gut microbiota and metabolites. It offers a deeper perception of the colonic inflammation-inhibiting function of TPs and provides a new orientation for the application of TPs-related products.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA679459.

ETHICS STATEMENT

The animal study was reviewed and approved by the guidelines of the Committee on Care and Use of Laboratory Animals of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences.

AUTHOR CONTRIBUTIONS

WP obtained financial support and conceived this study. YX designed the animal experiment and wrote the manuscript. LX, ZZ, and WZ performed the animal experiment, generated the tissue samples and prepared testing samples. XH, JT, and JZ analyzed the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.648162/full#supplementary-material

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Microbiota and Tuberculosis: A Potential Role of Probiotics, and Postbiotics

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Tuberculosis (TB), caused by *Mycobacterium tuberculosis* attacking the lungs and other organs, is one of the most common infectious disease worldwide. According to the WHO's 2020 report, a quarter of the world's population were infected with *M. tuberculosis*, and ∼1.4 million people died of TB. Therefore, TB is a significant public health concern, which requires cost-effective strategies for prevention and treatment. The microbiota has been considered as a "forgotten organ" and a complex dynamic ecosystem, which plays a significant role in many physiological processes, and its dysbiosis is closely associated with infectious disease. Recently, a few studies have indicated associations between TB and microbiota. This review summarizes studies concerning the alterations of the gut and respiratory microbiota in TB, and their relationship with host susceptibility to *M. tuberculosis* infection, indicating that microbiota signatures in different stages in TB progression could be considered as biomarkers for TB diagnosis and control. In addition, the potential role of probiotics and postbiotics in TB treatment was discussed.

Keywords: tuberculosis, microbiota, probiotics, postbiotics, immunity

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TUBERCULOSIS

Tuberculosis (TB), typically caused by *Mycobacterium tuberculosis* infection, is a highly communicable infectious disease (1). According to the WHO's 2020 report, in 2019, around 10 million people were infected with and developed TB, and 1.4 million deaths occurred (1). *M. tuberculosis* can be expelled by TB patients, spread through the air and infect others (2). Not only the lungs, other organs, such as the brain and spine can also be invaded by *M. tuberculosis* (1). At the onset of *M. tuberculosis* invasion, its cell wall components are recognized by pathogen-recognition receptors (PRRs), consequently activating the innate immune response (3). Antimycobacterial activity of alveolar macrophages is activated by tumor necrosis factor-alpha (TNF-α) and interferon gamma (IFN-γ). Immune cells, including macrophages, neutrophils, and T and B cells, migrate to infection sites, form granulomas around *M. tuberculosis*, and restrict its replication (latent TB infection) (4). At this point, *M. tuberculosis* can still survive and replicate in granulomas by inhibiting the maturation of phagolysosomes and destructing the patterns of cell death and immune response. However, when granulomas are impaired due to factors, such as HIV infection, smoking, aging, and malnutrition, *M. tuberculosis* can escape from granulomas and spread to other tissues (active TB infection) (5). Most individuals with *M. tuberculosis* invasion remain symptom-free (latent TB infection), and 5–10% of the ∼2 billion infected people will develop active TB, showing

symptoms, such as bad cough, fever, weight loss, chest pain, and night sweats (**Figure 1**). People with diabetes, alcohol intake disorder, HIV infection, and smoking have a higher probability of developing TB (6). TB typically requires extended treatment with broad spectrum antibiotics for 6-9 months, hence generally resulting in drug-resistant TB (1). Therefore, it is significant to introduce novel strategies to control TB and improve treatment outcome.

MICROBIOTA AND TUBERCULOSIS

The concept of human microbiota referring to "microbial community structure in habitats of the human body such as the skin, vagina, oral cavity, gut, and lower respiratory tract" was firstly introduced by Lederberg and McCray (7). The microbiota has been considered as a "forgotten organ" and a complex dynamic ecosystem, which plays a significant role in many physiological processes, including digestion and nutrient absorption, and regulation of the immune system (8). The gut microbiota equilibrium plays a positive role in systemic and lung immunity, through regulating the differentiation of T cells, migration and apoptosis of immune cells, activation of toll-like receptor signaling, and suppressing inflammatory tone (9). The pulmonary microbiota is also crucial to protect against pathogens, regulate Th1/Th2 immune response, and control inflammation (10). Therefore, human microbiota could play an important role against *M. tuberculosis* infection. Consequently, microbiota alterations may contribute to the spectrum of TB pathogenesis, and it is critical to characterize the human microbiota profile at every stage of *M. tuberculosis* infection, which may be helpful to achieve the goal of the End TB Strategy, introduced by WHO (11). Available studies concerning the gut and pulmonary microbiota in healthy and TB patients, effects of anti-TB treatment on microbiota, and the association between microbiota composition and TB treatment efficiency are summarized in **Table 1**.

Gut Microbiota Alterations in TB Patients

Gut microbiota plays a significant role in modulating the host immune system (6). Gut microbiota could be significantly changed due to pulmonary infection with influenza virus stemming from a mechanism dependent on type I interferons (26). Several studies have found that there were significant differences between TB patients and healthy controls in gut microbiota. In a cross-sectional research study, Hu et al. (12) characterized the gut microbiota profile in Chinese TB patients and found that *M. tuberculosis* infection led to a decreased α diversity, which was mainly associated with alterations in *Bacteroides* relative abundance. Another research group in China recruited 31 healthy controls and 46 TB patients, and observed significant declined microbiota diversity and number, characterized by a remarkable decline in short chain fatty acid (SCFA)- producing bacteria. Besides, single nucleotide

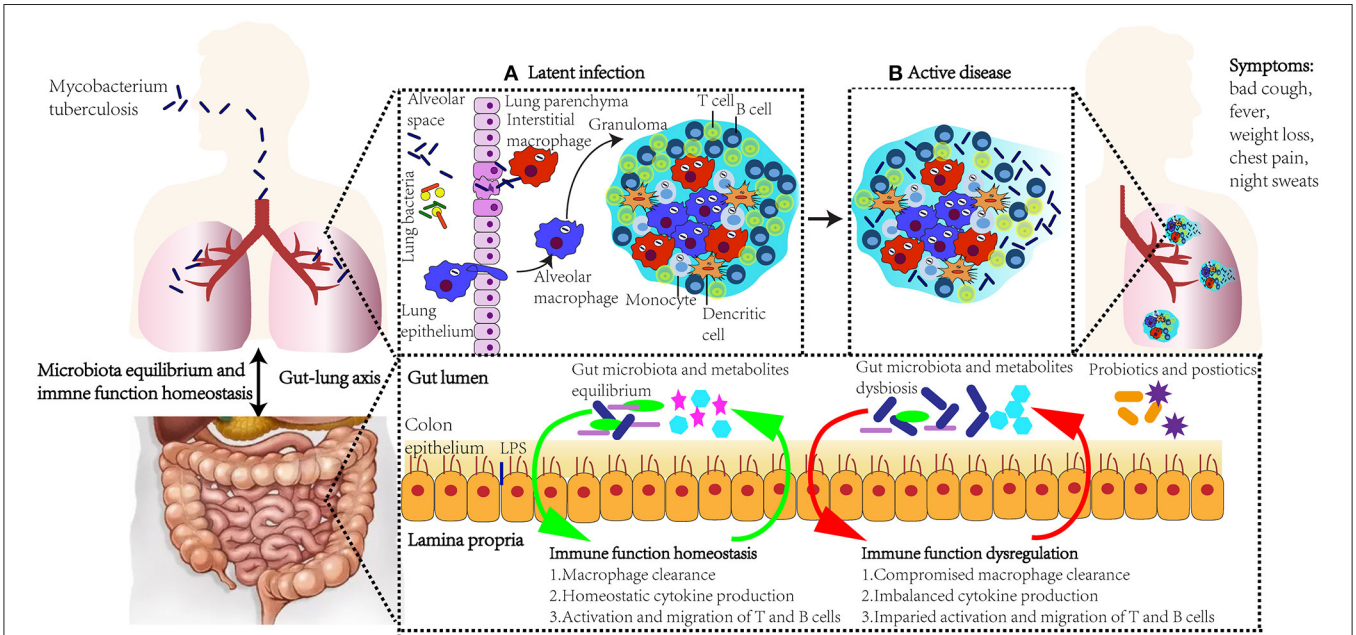

FIGURE 1 | The role of immune response, gut microbiota, lung microbiota, and gut–lung axis in *M. tuberculosis* infection. Upon exposure to *M. tuberculosis*, alveolar epithelial cells are the first cell lines to recognize and bind to the outer surface molecules of the mycobacteria. Immune cells, including macrophages and T and B cells, migrate to infection sites, form granulomas around *M. tuberculosis*, and restrict its replication **(A)**. However, when granulomas are impaired due to factors, such as HIV infection, smoking, aging, and malnutrition, *M. tuberculosis* escape from granulomas and spread to other tissues **(B)**. Active TB patients show symptoms such as bad cough, fever, weight loss, chest pain, and night sweats. The gastrointestinal tract and lung influence the microbiota and immune function homeostasis of each other, and this bidirectional gut-lung axis consequently influences the host immune response against *M. tuberculosis*. Novel supplementation, such as probiotics and postbiotics, could modulate microbiota and regulate immune function, and be applied as strategies in TB prevention and treatment.

TABLE 1 | Example of studies designed to determine the relationship between gut microbiota and tuberculosis.

Host/source	Sample size	Location	Methods	Results	References
Human	13 healthy controls, 28 TB patients, 23 TB patients anti-TB therapy	Shenzhen, China	Fecal samples, 16S rRNA sequencing, V4 region	*M. tuberculosis* infection led to a decreased α diversity, mainly associated with alterations in *Bacteroides* genus. During anti-TB treatment, genus *Clostridiales* significantly decreased, *Bacteroides* OTU230 and *Bacteroides fragilis* increased.	(12)
Human	31 healthy controls, 46 TB patients	Beijing, China	Fecal samples, metagenomic Sequencing	Declined microbiota diversity and number, remarkable decline in SCFAs -producing bacteria. SNPs in the species of *Bacteroides vulgatus* were dramatically different.	(13)
Human	20 healthy controls, 19 NTB, and 18 RTB	Chengdu, China	Fecal samples, 16S rRNA sequencing, V4 region	Actinobacteria and Proteobacteria were significantly higher, Bacteroidetes were lower in the RTB patients compared to controls. *Prevotella* and *Lachnospira* were dramatically lower in NTB and RTB compared to the control group.	(14)
Human	18 healthy children, 18 pediatric TB patients	Sichuan, China	Fecal samples, 16S rRNA sequencing, V3-V4 region	Declined microbiota diversity, increased abundance of *Prevotella*, *Enterococcus*, and reduced abundance of *Ruminococcaceae*, *Bifidobacteriaceae,* and *prausnitzii.*	(15)
Human	23 healthy controls, 25 TB patients	Taipei	Fecal samples, 16S rRNA sequencing, V3-V5 region	Decreased ratio of *Firmicutes* to *Bacteroidetes* in TB patients compared to healthy controls.	(16)
Human	16 healthy controls, 25 TB patients	New Delhi, India	Fecal samples, 16S rRNA sequencing, V6-V7 region	Firmicutes and Actinobacteria significantly increased in TB patients.	(17)
Female C57BL/6 mice, 6-7 week	CT: control mice; Abx: Abx treated mice; Mtb: mice infected by *M. tuberculosis*; Abx-Mtb: mice after treated with Abx, mice infected by *M. tuberculosis*; Mtb-INH: mice infected by Mtb and treated with INH; Abx-Mtb-INH: Abx treated mice infected with *M. tuberculosis* prior to INH therapy (n = 5 mice per group or cage).	Punjab, India	Fecal samples, quantitative real-time PCR	Abx led to decreased abundance of commensal bacteria *Campylobacter*, *Bifidobacterium,* and *Lactobacillus*, and increased abundance of *Enterococcus* and *Bacteroides*.	(18)
Human	52 healthy controls, 6 patients with MDR-TB treatment, 18 patients recovered from MDR-TB treatment	Linyi, China	Fecal samples, 16S rRNA sequencing, V3-V4 region	26% drop in microbiota diversity and significant changes in composition in patients with MDR-TB compared to controls. 16% drop in microbiota diversity and altered taxonomic composition in patients recovered from MDR-TB compared to controls.	(19)
Human	50 healthy controls, 19 TB patients with TB therapy	Port-au-Prince, Haiti	Fecal sample, 16S rDNA and metagenomic DNA sequencing	TB therapy deplete multiple immunologically significant commensal bacteria, such as *Ruminococcus, Eubacterium, Lactobacillus*, and *Bacteroides,* persist for at least 1.2 years.	(20)
Female mice of 4–8-week-old C57BL/6J-CD45a	CT: control mice; TB+HRZ: mice challenged with *M. tuberculosis* followed by HRZ therapy (*n* = 5 mice per group or cage).	Evanston, USA	Fecal sample, 16S rRNA sequencing, V3-V4 region	HRZ could immediately and reproducibly cause microbiota composition changes during the whole treatment course, and even 3 months after the treatment stop, with significant decreases in members of class Clostridia.	(21)
Human	10 healthy controls, 6 TB patients	PCTY Yucatán, Mexico	BAL samples, 16S rRNA sequencing, V3-V4 region	The diversity was decreased in TB patients compared to healthy volunteers, characterized by a significant decline in *Streptococcus* genus and increase in *Mycobacterium*.	(22)
Human	70 healthy controls, 70 TB patients,	Shenzhen, China	BAL samples, 16S rRNA sequencing, V3-V4 region	The α diversity was decreased in TB patients compared to healthy volunteers	(23)

(Continued)

TABLE 1 | Continued

Host/source	Sample size	Location	Methods	Results	References
Human	14 healthy controls, 22 TB patients	Hong Kong, China	Sputum sample, 16S rRNA sequencing, V1-V2 region	TB patients were characterized by *Proteobacteria*, and *Bacteroidetes*, while those of healthy controls were *Firmicutes*. In addition, the abundance of *Mogibacterium, Moryella*, and *Oribacterium* was increased significantly in the TB patients compared to controls.	(24)
Human	20 healthy controls, 25 NTB patients, 30 RTB patients, and 20 TB patients with treatment unsuccessful	Shanghai, China	Sputum sample, 16S rRNA sequencing, V1-V2 region	An increase in abundance of *Streptococcus, Gramulicatella* and *Pseudomonas*, and a decrease in abundance of *Prevotella, Leptotrichia, Treponema, Catonella*, and *Coprococcus* in TB patients compared those in the healthy controls.	(25)

SCFAs, short chain fatty acids; NTB, new tuberculosis patients; RTB, recurrent tuberculosis patients; Abx, broad-spectrum antibiotics; MDR-TB, multi-drug-resistant tuberculosis; BAL, bronchoalveolar lavage; HRZ, isoniazid-rifampin-pyrazinamide.

polymorphisms (SNPs) in the species of *Bacteroides vulgatus* were dramatically different in TB patients compared to heathy controls (13). A research group by Luo, Liu (14) divided their TB patients into new tuberculosis patients (NTB) and recurrent tuberculosis patients (RTB). Their results showed that Actinobacteria and Proteobacteria were significantly higher, while Bacteroidetes, containing a variety of beneficial commensal bacteria, were lower in the fecal samples of RTB patients. In addition, *Prevotella* and *Lachnospira* were dramatically lower in the NTB and RTB compared with the healthy control group. Similar results were also found in children with TB. A case-controlled study found that the gut microbiota profile in pediatric TB patients was characterized by declined microbiota diversity, increased abundance of *Prevotella* and *Enterococcus*, and reduced abundance of Ruminococcaceae, Bifidobacteriaceae, and *Faecalibacterium prausnitzii*, which are beneficial to host health (15). Another study found a decreased ratio of *Firmicutes* to *Bacteroidetes* in TB patients compared to healthy controls using 16S rRNA sequencing (16). A research group in India applied 16S rRNA sequencing to distinguish microbiota composition between TB patients and healthy controls, and found that Firmicutes and Actinobacteria were significantly increased in TB patients (17). According to these studies, a distinct gut microbiota profile could be observed between healthy and TB patients, and microbiota signatures in different stages in TB progression could also be identified. However, the causal relationship and whether an altered gut microbial profile with declined bacterial diversity increases the susceptibility to TB needs to be further investigated.

Effects of Anti-TB Treatment on Gut Microbiota

Antibiotic administration could critically affect gut microbiota, leading to the disruption of bacterial equilibrium (27). Broad-spectrum antibiotics could decrease richness, diversity, and evenness of the whole bacteria community, and after antibiotics, gut microbiota could either recover or achieve a new balance (28). The impact of antibiotics used in TB treatment on gastrointestinal microbiota has also been investigated. Hu et al. (12) found that anti-TB therapy could lead to rapid, dramatic changes in the diversity and composition of the microbiota community. During anti-TB treatment, the relative abundance of genus *Clostridium* significantly decreased, whereas several members of the *Bacteroides* genus, such as *Bacteroides fragilis* and *Bacteroides* OTU230 increased. Besides, after 1 week of TB treatment, OTU8 and OTU2972 assigned to the family Erysipelotrichaceae strikingly increased, whereas the rest of the Erysipelotrichaceae family declined. Similarly, Negi, Pahari (18) applied an *in vivo* mouse model and found that broad-spectrum antibiotics could lead to significant alterations in gut microbiota composition with decreased abundance of commensal bacteria *Campylobacter*, *Bifidobacterium,* and *Lactobacillus*, and increased abundance of *Enterococcus* and *Bacteroides*. Multiple antibiotics in the case of TB treatment could not only result in immediate dramatic changes in gut microbiota composition but also even after a long

period of recovery. Wang, Xiong (19) found that multi-drug-resistant tuberculosis (MDR-TB) treatment could result in changes in gut microbiota with a 26% decline in microbiota diversity and a significant change in microbiota composition. In addition, there was a 16% decrease in gut microbiota community richness in recovered patients from MDR-TB treatment compared to the untreated group. However, this study only recruited six MDT-TB treated volunteers, hence interpretation of these results should be cautious. These results were in agreement with previous studies. Wipperman, Fitzgerald (20) found that TB therapy could dramatically deplete multiple immunologically significant commensal bacteria, such as *Ruminococcus, Eubacterium, Lactobacillus*, and *Bacteroides*. These microbiota alterations could even persist for at least 1.2 years. Using an *M. tuberculosis*-infected mouse model, Namasivayam, Maiga (21) found that isoniazid-rifampin-pyrazinamide treatment could immediately and reproducibly cause microbiota composition changes during the whole treatment course, and even 3 months after the end of treatment, with significant decreases in members of class Clostridia, such as *Acetivibrio, Robinsoniella, Alkaliphilus, Stomatobaculum, Butyricicoccus*, *Acetanaerobacterium, Tyzzerella, Ruminococcus*, and *Peptococcus*.

Gut Microbiota Alterations and Anti-TB Treatment Efficiency

Several diseases such as cancer, allergies, autoimmune diseases, and infections, could be triggered and aggravated by altered gut microbiota composition (29, 30), highlighting the significance of gut microbiota in treatment efficiencies in hosts. A few studies have indicated that altered microbiota balance could limit the potency of anti-TB drugs and TB treatment efficiency. Negi et al. (18) applied an *in vivo* mouse model to study the influence of gut microbiota dysbiosis on isoniazid (INH) efficiency against *M. tuberculosis*, and found that a declined abundance of *Lactobacillus*, *Bifidobacterium*, and *Campylobacter* caused by antibiotic pre-treatment could lead to immune response impairment to INH treatment in *M. tuberculosis* clearance and more serious granulomatous development. In addition, this group also demonstrated that impairment of the intestinal innate defense and immunity stemmed from microbiota changes during INH therapy, and resulted in lower levels of antimicrobial peptide RegIII γ and pro-inflammatory cytokines TNF-α and IFN-γ, and higher levels of anti-inflammatory cytokine IL-10. Khan, Mendonca (31) also found that intestinal changes with increased abundance of *Bacteroides* and *Verrucomicrobiaceae*, and decreased abundance of *Lachnospiraceae* compromised alveolar macrophage immune function to *M. tuberculosis*. Similar results were found previously. Dumas et al. (32) demonstrated that reduced Bacteroidetes and Firmicutes, and increased Proteobacteria of antibiotic pre-treatment mice was associated with an increased early lung colonization by *M. tuberculosis*, and indicated the role of microbiota in contributing to early protection possibly through sustaining the functions of mucosal-associated invariant T cells. These findings suggest a role of gut microbiota in anti-TB treatment. Microbiota alterations could impair anti-TB treatment in *M. tuberculosis* survival and clearance. In addition, gut microbiota could influence the pharmacokinetics of anti-TB drugs, through producing enzymes which can activate or inactivate drugs, and binding to drugs hence influencing bioavailability (33). Therefore, modulating gut microbiota and maintaining equilibrium using probiotics and postbiotics could enhance the efficiency of anti-TB drugs and improve host immunity against *M. tuberculosis*.

Pulmonary Microbiota Alterations in TB Patients

The taxa of pulmonary microbiota have been demonstrated to be similar to those along the respiratory tract, with a declined microorganism burden compared with those in the oral cavity (34). There is a balance of dynamic bacterial community shift along the respiratory tract, which results from gastric content aspiration, mucosa dispersion and elimination, coughing, and immunity (35). However, if this equilibrium is disrupted, the microbiota community would significantly change and be associated with lung disease (36, 37). A few studies have described the pulmonary microbiota composition in TB patients, some collected bronchoalveolar lavage (BAL) samples, and some collected sputum samples. BAL samples are mainly used to describe microbiota colonized in the lower respiratory tract, although they are difficult to collect and are potentially contaminated by oral microbiota. Vázquez-Pérez et al. (22) used BAL samples to describe and compare the pulmonary microbiota composition in TB patients and healthy volunteers. Using 16S rDNA sequencing methods, the diversity of microbiota was found to be decreased in TB patients compared to healthy volunteers, characterized by a significant decline in *Streptococcus* genus and increase in *Mycobacterium*. Similar results have also been demonstrated in Chinese TB patients by Hu et al. (23), with lower α diversity of pulmonary microbiota composition in BAL in TB patients. Meanwhile, a few studies also investigated the respiratory microbiota profile in sputum samples which are non-invasively and more easily collected compared to BAL samples. After comparing sputum samples from 22 TB patients and 14 healthy controls, Cheung et al. (24) found that the pulmonary microbiota of TB patients were characterized by Proteobacteria and Bacteroidetes, while those of healthy controls harbored Firmicutes. In addition, the abundance of *Mogibacterium, Moryella,* and *Oribacterium* were increased significantly in the TB patients compared to controls. A research group by Wu et al. (25) further divided TB patients into NTB, RTB, and treatment failure TB patients, and compared their sputum samples. They showed an increase in abundance of *Streptococcus, Gramulicatella,* and *Pseudomonas*, and a decrease in abundance of *Prevotella, Leptotrichia, Treponema, Catonella,* and *Coprococcus* in TB patients compared those in the healthy controls. The results also showed that RTB had higher *Pseudomonas /Mycobacterium* and lower *Treponema/Mycobacterium* ratios compared to NTB. Besides, the abundance of *Pseudomonas* and *Pseudomonas*/*Mycobacterium* increased in patients whose TB treatment were unsuccessful compared to NTB. According to these studies, pulmonary

microbiota diversity is observed to be decreased in TB patients compared to healthy controls. In addition, the dominant lung microbiota species are different between TB patients and healthy controls. These findings also suggest the important role of pulmonary microbiota in TB pathogenesis and treatment efficiency, consequently more attention should be paid to pulmonary microbiota for improving TB control strategies and treatment efficiencies in the future.

POTENTIAL ROLE OF PROBIOTICS AND POSTBIOTICS

As mentioned above, gut microbiota alterations could impair the function of macrophages and disrupt the activation of immune cells in *M. tuberculosis* clearance. Therefore, supplementation which can modulate gut microbiota and maintain equilibrium could be applied to improve host immunity against *M. tuberculosis*, and enhance the treatment outcome of anti-TB drugs.

In the early twentieth century, it was hypothesized by Metchnikoff that the long life span of Bulgarian peasants resulted from their large intake of fermented milk which contained beneficial bacteria, and the term probiotic was initially proposed (38). In 2014, probiotics were stipulated as "defined contents, appropriate viable count at end of shelf life, and suitable evidence for health benefits," and the safety of probiotics were addressed by Hill (39). Probiotics have been shown to modulate microbiota composition through inhibiting growth and activity of harmful bacteria and pathogens, and stimulating those of beneficial bacteria (40). Furthermore, probiotics can modulate the host immune system through stimulation of host immunoglobulins and antibacterial compounds, and enhancement of the innate and adaptive immune response (41). In an *in vitro* study, probiotic bacteria *Lactobacillus brevis*, *L. plantarum*, and *L. fermentum* showed antimicrobial activity against *M. tuberculosis* (42). In another *in vitro* study, probiotic *L. casei*, *L. plantarum*, and *L. salivarius* showed strong antimicrobial activity against *M. bovis* Bacillus Calmette-Guerin (BCG), and this anti-mycobacterial activity may have stemmed from the metabolites produced by the *Lactobacillus* species, which harbor genes encoding for class II bacteriocins and bacteriolysins. Furthermore, *L. plantarum* significantly decreased BCG intake by phagocytes, whereas *L. casei* increased BCG intake and *L. salivarius* had no effect on it (43). The inhibitory activity against *M. tuberculosis* by lactobacilli is in agreement with a previous study (44). In an *in vivo* mouse model, Negi et al. (45) found that a decrease in Bacteroidetes and Firmicutes, and an increase in Proteobacteria caused by antibiotics could result in the declined expression of macrophage-inducible Ca^{2+}-dependent lectin receptor (mincle), which functions as a pattern recognition receptor recognizing and binding to the carbohydrate structure on pathogens including those on *M. tuberculosis*, and subsequently induce an innate immune response (46). In addition, gut microbiota alterations lead to increased burden of *M. tuberculosis*, a decreased effector and memory T cell population, and increased regulatory T cells in the lungs (45). However, probiotic supplementation with *Lactobacillus plantarum* MTCC 2621 could restore mincle and MHC-II expression on lung dendric cells, reduce lung *M. tuberculosis* burden, decrease regulatory T cells, and increase activated and effector memory CD4T cells exhibiting a CD44hi phenotype and a CD62LloCD44hi phenotype, respectively (45). This study indicated that the functions of lung dendric cells and T cell against *M. tuberculosis* in dysbiotic mice could be enhanced by probiotic *L. plantarum*. Although few studies showed the antagonistic and immunoregulatory effects against *M. tuberculosis*, these findings highlight the potential role of probiotics as a novel strategy in TB treatment.

The concept of postbiotics is proposed according to the findings that beneficial effects of bacteria are modulated by secreted metabolites. Postbiotics are inactivated microbial cells and/or their components that confer beneficial effects on host health (47). Microbial cell-wall fractions, extracellular or surface-associated proteinaceous molecules, exopolysaccharides, or microbial metabolic such as SCFA, vitamins, amino acids, peptides, etc, which could exert benefits to host health, directly or indirectly belong to postbiotics (48). Khusro et al. (49) purified and characterized an anti-tubercular protein produced by strain *Staphylococcus hominis* MANF2, with molecular mass 7712.3 Da. In addition, they found this inhibition effect was dose-dependent. Carroll et al. (50) found that lacticin 3147, an antimicrobial peptide produced by *Lactococcus lactis* subsp. *cremoris* MG1363, strongly inhibited the growth of *M. tuberculosis* H37Ra *in vitro*, with an MIC_{90} value of 7.5 mg/L, and demonstrated its greater potential as a therapeutic agent. Another antimicrobial protein produced by *Lactococcus lactis* subsp. *lactis* was also found to act against mycobacteria, which is associated with proton motive force collapse and intracellular ATP decrease (51). Indole propionic acid, a gut microbiota metabolite was also identified as an anti-tubercular agent (52–54). Negatu et al. (54) primarily screened 1,000 fragments in the Maybridge Ro3 library, and identified 29 compounds *in vivo* with the most anti-tubercular activity. Subsequently, 29 compounds were co-cultured with *M. tuberculosis* to determine their bactericidal activity against *M. tuberculosis*, and half of them could reduce *M. tuberculosis* viability 100-fold. Among these compounds, indole propionic acid showed the strongest inhibition effect against *M. tuberculosis*. Consequently, it was tested in a mouse model, which were infected with a low dose of *M. tuberculosis* by the aerosol route, and found to reduce bacterial load in spleen seven-fold, indicating its direct anti-tubercular activity. This research group further focused on the antibacterial mechanism of indole propionic acid. After metabolic, chemical rescue, genetic, and biochemical analyses, they found indole propionic acid could mimic physiological allosteric inhibitor of TrpE, block tryptophan biosynthesis in *M. tuberculosis*, and hence show antimycobacterial activity (53). These findings illustrate the potential anti-tubercular activity of postbiotics, although more research needs to be performed to elucidate microbiota and host factors involved in anti-tubercular activity, the role of postbiotics in TB susceptibility, progression

and severity, and the application of postbiotics in anti-TB treatment.

THE GUT–LUNG AXIS IN TB

Host systemic and lung immunity plays an important role in TB pathogenesis through controlling the clearance, survival, and replication of *M. tuberculosis* (55). Upon exposure to *M. tuberculosis*, alveolar epithelial cells are the first cell lines to recognize and bind to the outer surface molecules of mycobacteria through several types of PRRs, such as C-type lectins, and TLRs (56). Subsequently, several signaling pathways are activated to induce the secretion of cytokines and chemokines, and to initiate the migration of immune cells to the infection sites (55).

There are increasing numbers of studies illustrating the role of gut and pulmometry microbiota in modulating immune function in prevention, progression, and treatment of chronic respiratory diseases (57–61). The microbiota influences TB prevention, pathogenesis, and treatment mainly by affecting the percentage and function of immune cell subsets, producing bacteriocins and bacteriolysins that restrict the growth of *M. tuberculosis* directly, and/or by influencing bioavailability and pharmacokinetics of anti-TB drugs (62). Gut microbiota equilibrium has been shown to play an important role in regulating immune response through improving immune cell response against *M. tuberculosis* and promoting Th1/Th2 balance (31, 63, 64). Innate immune cells could be affected directly by gut microbiota and their metabolites, or indirectly by cytokines secreted by epithelium cells or dendritic cells, which in turn activate the migration of adaptive immune cells to infection sites (6). An altered gut microbial balance could lead to the suppressed ability of dendritic cells in antigen presentation, which consequently result in a diminished innate and adaptive immune response against *M. tuberculosis* (18). Expression of C-type lectins, a type of PRR would also be reduced by gut microbiota alterations, hence exerting an adverse effect on immune cell activation and *M. tuberculosis* clearance (45). In addition, gut microbiota alterations with increased abundance of *Bacteroides* and *Verrucomicrobiaceae*, and decreased abundance of *Lachnospiraceae* would result in a compromised anti-TB immune response with elevated numbers of T regulatory cells which increase susceptibility to TB, and decreased numbers of Th1 cells which promote protective immunity against *M. tuberculosis* (65). Gut microbiota metabolites could be produced and secreted into the bloodstream, and then transported to the lungs, thus stimulating the local immune response. Gut microbiota metabolites, such as butyrate and propionate, could decrease the lung production of IL-17, suppress Th1 immunity, and increase numbers of T regulatory cells, consequently influencing the outcome of *M. tuberculosis* infection (66–68). Another gut microbiota metabolite, indole propionic acid, could disturb tryptophan biosynthesis in *M. tuberculosis*, and hence inhibit its growth directly (53, 54). Lung microbiota also plays a key role in local immunity through affecting recruitment and activation of epithelial cells and T regulatory cells (69). The phylum Bacteroidetes has been shown to downregulate lung inflammatory status (70), whereas *Prevotella* spp. and *Veillonella* spp. could upregulate lung inflammatory status mediated by Type 17 helper T cells (71). In addition, respiratory commensal bacteria *Corynebacterium pseudodiphtheriticum* has been shown to improve the function of alveolar macrophages, and regulate the innate immune response against virus infection, indicating the potential role of *C. pseudodiphtheriticum* as a next-generation probiotic (72). There is a strong association between lung microbiota and gut microbiota; they overlap in microbiota composition, and microbiota diversity in two organs decrease or increase simultaneously (73). The alterations in gut microbiota could affect lung microbiota, which would influence lung inflammatory response and granuloma formation upon *M. tuberculosis* infection (10, 74). Meanwhile, the composition of lung microbiota also affects gut microbiota through translocation of microorganisms into blood (75). Therefore, the gastrointestinal tract and lung could influence the microbiota and immune function homeostasis of each other, and this bidirectional gut-lung axis consequently could influence the host immune response against *M. tuberculosis*. Novel supplementation, such as probiotics and postbiotics, could modulate microbiota and regulate immune function through competing with pathogenic bacteria, conferring antibacterial effects, regulating innate immune response, stimulating epithelial cell growth, and improving barrier function (76). Therefore, they could be applied as strategies in TB prevention and treatment.

CONCLUSIONS

The gut-lung axis plays an important role in TB prevention and treatment outcome, through affecting host immune response against *M. tuberculosis*. Several studies have suggested that microbiota could play a significant role in TB pathogenesis and treatment efficiency, the dysbiosis of microbiota may result in adverse impacts on immune response to *M. tuberculosis* infection, a more serious development of granulomatous, and decreased efficiencies of anti-TB drugs. Meanwhile, multi-drugs used in TB treatment could significantly alter the gut and pulmonary microbiota community for a long time. Therefore, the microbiota becomes an inevitable subject in TB research area, and the identification and validation of microorganisms contributing to TB progression and treatment outcomes in epidemiologically representative populations should be undertaken. In addition, probiotics and postbiotics have exhibited anti-tuberculosis activity *in vitro* and *in vivo*, indicating their potential for application in anti-TB treatment to overcome complications caused by the current use of multiple antibiotics. Some members of respiratory commensal bacteria also show the potential to be used as next-generation probiotics in resistant respiratory infection. In summary, the microbiome will contribute to TB therapy efficiency, and the application of probiotics and postbiotics could be explored as an add-on to current therapies, or drug optimization strategies.

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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The Impact of Dietary Sphingolipids on Intestinal Microbiota and Gastrointestinal Immune Homeostasis

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The large surfaces of gastrointestinal (GI) organs are well adapted to their diverse tasks of selective nutritional uptake and defense against the external environment. To maintain a functional balance, a vast number of immune cells is located within the mucosa. A strictly regulated immune response is required to impede constant inflammation and to maintain barrier function. An increasing prevalence of GI diseases has been reported in Western societies over the past decades. This surge in GI disorders has been linked to dietary changes followed by an imbalance of the gut microbiome, leading to a chronic, low grade inflammation of the gut epithelium. To counteract the increasing health care costs associated with diseases, it is paramount to understand the mechanisms driving immuno-nutrition, the associations between nutritional compounds, the commensal gut microbiota, and the host immune response. Dietary compounds such as lipids, play a central role in GI barrier function. Bioactive sphingolipids (SLs), e.g. sphingomyelin (SM), sphingosine (Sph), ceramide (Cer), sphingosine-1- phosphate (S1P) and ceramide-1-phosphate (C1P) may derive from dietary SLs ingested through the diet. They are not only integral components of cell membranes, they additionally modulate cell trafficking and are precursors for mediators and second messenger molecules. By regulating intracellular calcium levels, cell motility, cell proliferation and apoptosis, SL metabolites have been described to influence GI immune homeostasis positively and detrimentally. Furthermore, dietary SLs are suggested to induce a shift in the gut microbiota. Modes of action range from competing with the commensal bacteria for intestinal cell attachment to prevention from pathogen invasion by regulating innate and immediate defense mechanisms. SL metabolites can also be produced by gut microorganisms, directly impacting host metabolic pathways. This review aims to summarize recent findings on SL signaling and functional variations of dietary SLs. We highlight novel insights in SL homeostasis and SL impact on GI barrier function, which is directly linked to changes of the intestinal microbiota. Knowledge gaps in current literature will be discussed to address questions

relevant for understanding the pivotal role of dietary SLs on chronic, low grade inflammation and to define a balanced and healthy diet for disease prevention and treatment.

Keywords: sphingolipids, nutrition, immune modulation, gastrointestinal barrier, gastrointestinal microbiota, immunonutrition

INTRODUCTION

GI diseases are common in Western societies and associated with an increase in prevalence (1, 2). Estimations suggest 11% of the United States population (3) and 5-15% of the European population (with variations between Western Europe and Eastern Europe) (4, 5) are suffering from digestive diseases. This includes cancerogenic diseases affecting esophagus, stomach, intestines and pancreas, as well as inflammatory diseases like inflammatory bowel disease (IBD), esophagitis, coeliac disease, diverticular disease, alcoholic liver disease, acute and chronic pancreatitis and many more (5). Moreover, digestive disorders have been associated with psychosomatic manifestations, such as fatigue/neurasthenia, anxiety, phobia and panic disorders and pain syndromes (2). Although, current literature suggests to combine different scientific disciplines to identify functional relationships between immune, inflammatory and neurological disorders, analysis approaches involving data sets of the different scientific disciplines are limited. The increased prevalence of GI diseases is a major challenge for our health care systemassociated with increasing costs.

Recent demographic data suggest Western lifestyle to play a causative role in disease prevalence. In search for explanations of this phenomenon, investigators are increasingly focusing on the gut and its immune system. It is suggested that low grade and chronic inflammation rather than an acute defense reaction, might be the cause for disease development by slowly altering the immune response (6, 7). An inflammation causing a systemic response in the whole body would have effects on multiple organ systems. Besides manipulating the environment of affected tissue types towards tumorigenesis, a long lasting, low grade inflammation can also result in common chronic conditions, such as allergic diseases, autoimmunity, arteriosclerosis, obesity, insulin resistance and depressive disorders. By focusing on the gut as source of inflammation it is paramount to understand the role of nutrition on the cross-talk between the microbiome, GI barrier function and the gut associated lymphoid tissues (GALT). Western high fat diets have been reported to enhance intestinal inflammation by promoting gut permeability and altering gut microbiota (8, 9). In response, dietary modulation reduces severity of GI symptoms related to cancer, allergy and autoimmunity and has been suggested as a simple and commonly available approach to counteract disease onset and progression (10–12). Nutritional lipids have received much attention in the field of immuno-nutrition. Lipids are not only considered as energy storage molecules but have also been reported to be involved in the regulation of cell migration, the production of hormones and to act as second messenger molecules. These characteristics enable lipids to modulate immuneresponses (13, 14).

SLs are a highly diverse lipid class found in cellular membranes, lipoproteins and other lipid-rich structures, such as the skin. Their metabolites influence apoptosis, cell growth and cell migration. SLs contribute to pro- and anti-inflammatory immune responses. These lipid molecules are produced endogenously and their metabolism is strictly regulated. SLs are also found in food products, ingested and absorbed in the GI tract affecting its immune activation status and subsequently inflammatory and inflammation-related diseases. Previously reported mechanisms of SL action are inhibition of intestinal lipid uptake (15, 16), activation of pro- and anti-inflammatory receptors (17), lymphocyte chemotaxis (18), neutralization of bacterial endotoxins (19) and alterations of the intestinal microbiota (20–22). In context with the demographic changes of the last decades, the increasing prevalence of "civilization diseases" in Western societies and diet specific differences in SL content and composition, we suggest that dietary SLs contribute to the regulation of inflammatory stimuli.

Therefore, elucidating intestinal SL pathways and effector metabolism is crucial for identifying novel key players in immuno-nutrition to combat a dysregulated GI immune response.

A BRIEF INTRODUCTION TO SLs

Johann Ludwig Wilhelm Thudichum was the first who described SLs after identifying them as constituents in brain tissue. Due to their highly enigmatic nature, he named them after the ancient sphinx (23). The large and complex metabolism, the enormous amount of SL species and the lipophilic character of SLs have been huge obstacles for scientists. With the progress in genetic, molecular and technical methods during the past decades, a more lipid and enzyme centered approach was possible for investigations. Analysis of their detailed molecular structure and SL content of cells, tissues and food products is now possible.

SL Structures

SLs are a structurally highly diverse lipid class with over 4000 distinct SL subtypes (24). While most common in eukaryotic organisms, they are rarely found in prokaryotes (e.g. *Bacteroidetes* and *Proteobacteria*), archaea and viruses (25). Originally, SLs were described as components of cell membranes forming, together with cholesterol, phospholipids and proteins, membrane microdomains called lipid rafts, which are important for cell signaling pathways (26). SL metabolites

emerged in studying inflammation, since their bioactivity was reported to regulate cellular signals involved in apoptosis and cell viability. SLs consist of a fatty acid linked to the amino group of a sphingoid base and a headgroup associated with a hydroxyl head group of the base (**Figure 1**). The sphingoid base sphingosine (Sph) is the most common SL backbone in mammals. However, different organisms display more than one single sphingoid backbone type. Human SLs, for example, have mostly Sph as backbone, but also sphinganine, 4- hydroxysphinganine, as well as small amounts of longer chain length homologs (27). Plant sphingoid backbones often display more double bonds along the alkyl chain than mammalian SLs. Cers are formed when a Sph backbone is linked to a fatty acid, typically with a length of 16 – 26 carbon atoms, without a head group. Usually, the term "Cer" refers to N-acylsphingosines. However, some studies do not distinguish Cers by their sphingoid backbones and all N-acyl-sphingoid bases are called Cers (27). By adding different head groups, such as phosphocholine, sugars or more complex carbohydrates, the SL types gain different identities and functions (28). The linkage to a head group, which consists of a phosphate group esterified to an alcohol, like phosphocholine or phosphoethanolamine, to Cers results in synthesis of the phosphosphingolipid sphingomyelin (SM). Glycosphingolipids are formed by linking the sphingoid backbone to a carbohydrate head group. This subfamily consists of the most diverse SL types and based on their carbohydrate compositions they can be either neutral or acidic. By associating with a monosaccharide like glucose or galactose, neutral cerebrosides are formed. Linkage to more than one saccharide forms neutral globosides. Binding of oligosaccharides, N-acetylglucosamine, N-acetylgalactosamine and one or more sialic acid residues on the sugar chain forms gangliosides.

SL Metabolism and Catabolism

Although mammalian SLs consist of various species, their synthetic and catabolic pathways are shared. *De novo* synthesis of Cer starts with the condensation of a serine and palmitoyl-CoA to 3- ketosphinganine. The reaction is carried out by an enzyme called serine palmitoyltransferase. 3- ketosphinganine is then further reduced to sphinganine and acylated to dihydroceramide by one of six mammalian Cer synthases. Each of the Cer synthases has its own preferred acetyl-CoA substrate.

By introduction of a double bond at the sphingoid base a Cer is formed, which represents the branch point of SL metabolism. Cer synthesis takes place at the cytosolic leaflet of the endoplasmic reticulum (ER) (24). For further and more complex metabolism, Cers have to be transported to the Golgi apparatus. Here different head groups are added to the Cers. This results in formation of SM by the enzyme SM synthase, or to glycosphingolipids, such as cerebrosides and gangliosides, by respective enzymes. Additionally, Cer can also be produced by turnover of these more complex SLs i.e. *via* hydrolysis of SM and glycosphingolipids. Lastly, Cer is formed by recycling of SL metabolites, such as S1P or C1P, in the salvage pathway.

The most intensely studied catabolic pathway starts with the hydrolysis of SM to phosphocholine and Cer by alkaline, neutral and acid sphingomyelinases (SMases) (29, 30). Alkaline SMase is most abundant in the plasma membrane and endosomes of the microvilli enterocytes and works optimally at pH 8.5 – 9.

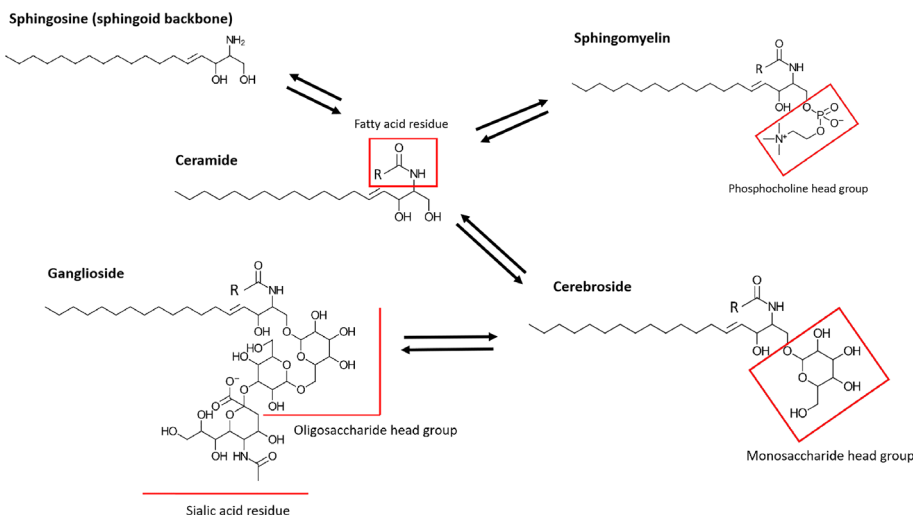

FIGURE 1 | Simplified structures of prominent human SLs. SLs consist of a sphingoid backbone, which is represented by Sph in this figure. By linking a fatty acid residue *via* an amide linkage, Cer (N-acylsphingosine) is formed. Cers differ depending on length and saturation of their alkyl chain on the fatty acid residue. The further addition of a head group, such as phosphocholine or phosphoethanolamine, to a Cer results in the formation of SM. More complex glycolsphingolipids are generated by addition of carbohydrate head groups. Neutral cerebrosides are formed by linkage with monosaccharide head groups. Linkage to oligosaccharide headgroups with one or more sialic acid residues forms gangliosides.

Noteworthy, differing from neutral and acid SMases, the alkaline SMase is restricted to the intestinal mucosa and to the liver in humans, where its activity is much higher than those of the other SMases. As an ectoenzyme, alkaline SMase is located on the cell surface and can be released into the lumen by both bile salt and pancreatic trypsin. Neutral SMases work preferentially at pH 7.5 and are located at the ER, nucleus, Golgi apparatus, plasma membranes and mitochondria. Acid SMases function below pH 5.5 and are mainly found in lysosomes. It can also be secreted by mast cells in response to inflammatory stimuli (31). After the hydrolysis, Cers can be either converted into ceramide-1-phosphate (C1P) by Cer kinase or be further degraded by one of six mammalian ceramidases. As a result, Sph and fatty acids (FAs) are produced. Endogenous Sph production is restricted by the breakdown of Cers (24). Furthermore, SM and Cer cannot be absorbed by enterocytes, in contrast to Sph and its metabolite S1P. S1P is generated by the phosphorylation of Sph by Sph kinase (SphK)1 and 2 (18, 32) and has two possible metabolic fates. It can either be dephosphorylated back to Sph by S1P phosphatases or degraded irreversibly by S1P lyase to phosphoethanolamine and hexadecenal, which will be used for acyl-CoA synthesis.

The Impact of Dietary SLs

In the last decades investigations on the increasing prevalence of diseases common for developed societies have focused on the impact of westernized dietary habits. Nutritional patterns including the frequent intake of high levels of protein, sugar, fat, salt and cholesterol have been suggested to promote chronic GI inflammations affecting immune responsiveness and subsequent diseases in multiple organ systems. With only minor amounts of a few micromoles per kilograms in fruits to several millimoles per kilogram found in dairy products, eggs and soybeans, the SL content varies enormously among nutritional compounds (25). On average, adults on a Western diet consume 0.3-0.4 g SLs per day mainly derived from SM (33). In Asian diet a lower amount of milk SM and much more cerebrosides are ingested (34). In the Western population, the intake of plant SLs is estimated to be 50 mg/day, although it can be much higher for vegetarians. Dietary SLs require a luminal breakdown to sphingoid backbones before intestinal absorption (35, 36) and differ in their structure depending on their origin (plant or animal SLs) (**Figure 2**). Various food products have been analyzed regarding their overall SL content [a detailed list is provided by Vesper et al. (25)]. However, the exact SL composition of different food products and diet-specific differences are still not well examined. Enhanced accessibility to high performance liquid chromatography (HPLC), gas chromatography-mass spectroscopy (GC-MS) and matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) approaches enable analysis of SL structure and quantity in different food sources (**Table 1**). First results have been highly interesting as they provide a more detailed insight into SL homeostasis. The uptake of plant sphingoid bases has been recently demonstrated to be less efficient in the small intestine. A study in rats suggested an efflux mechanism which allows enterocytes to release plant sphingoid bases back into the lumen (44). Despite possible effects on the host SL metabolism, higher amounts of plant Sph, together with non-digested SM and non- degraded Cer, is released into the colon. Possible downstream effects on the microbial gut community remain to be elucidated. Most SLs of mammalian food products consist of various SL types, such as SMs, cerebrosides, gangliosides, globosides or sulfatides. They are linked to a broad spectrum of different head group components (45). Plant SLs mainly consist of cerebrosides and phosphoinositides with glucose, galactose, mannose and inositol (46). Although, dietary SLs are not required for survival, they influence the composition of the gut microbiome and affect subsequent immune responses in the GI tract. By exploring underlying mechanisms, SL homeostasis has been suggested as a universal stress response. Considering the ongoing demographic changes, the healthcare system will be confronted with a huge burden. In view of the Western diet as an initial trigger for disease onset, a change in diet is a cheap, commonly accessible and most powerful tool to combat overall morbidity and increasing health care costs. Identifying diet-specific differences in SL composition is of uttermost importance to elucidate the accompanying role of SL metabolism in health and disease.

SLs AND INFLAMMATION

SL metabolites are well known to play a pivotal role in inflammatory signaling pathways. Low grade inflammation initiated by a Western diet cannot be detected *via* commonly available clinical biomarkers. However, constant stress and activation stimuli are suggested to be powerful promotors of metabolic changes (24, 47). Besides the detrimental impact of Western diet on health, a well-balanced nutrition might have a preventive and therapeutic function.

Sphingomyelin

SM is an important constituent of cell membranes. It is associated with cell signaling by the production of lipid-soluble second messenger molecules and by the formation of lipid rafts. It influences apoptosis through the degradation to Cers. SM has been discussed as a dietary modulator of cholesterol synthesis. By establishing a complex network of H-bonds, it interferes with cholesterol absorption slowing down its transfer to enterocytes. Via the same mechanism, it is suggested to interfere with triglycerides and FAs reducing serum and hepatic lipid concentrations in a dose dependent manner (48, 49). Investigations reported different inhibition patterns of intestinal cholesterol absorption when administering egg or milk SM. A stronger inhibitory effect of milk SM was suggested to cause a higher degree of saturation of fatty acyl groups (15, 16). An investigation on milk SM deriving from milk fat of Holstein cows and Jersey cows identified differences in SM content, which may be induced by breed, diet and stage of lactation (37). Similar results were found for analyses of soybean SLs. Significant differences in overall glucosylceramide (83.4–397.6 nmol/g) and major Cer (8.4–20.7 nmol/g) contents have been detected by comparison of 15 soy bean lines (50).

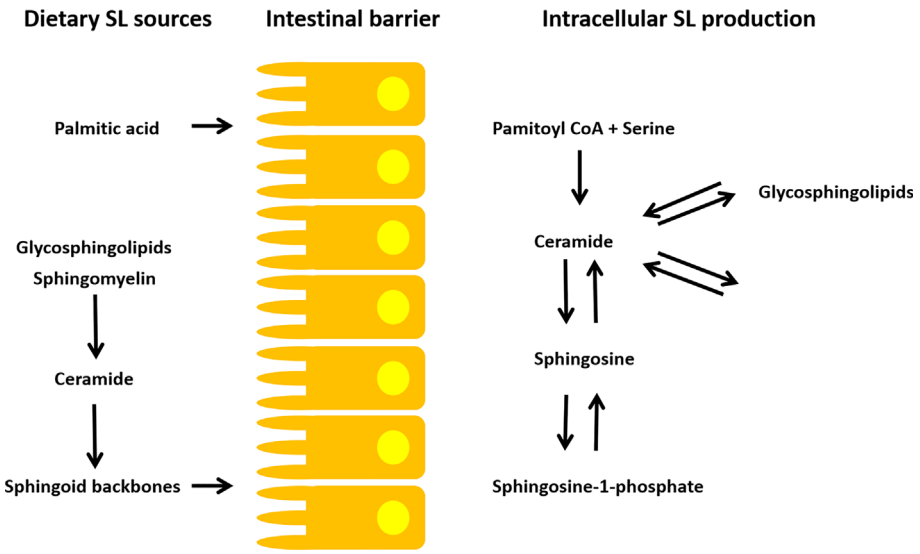

FIGURE 2 | SL metabolism of dietary and intracellular SLs. A luminal breakdown to sphingoid backbones is required for intestinal absorption of exogenous SLs. Additionally, dietary palmitic acid consumption is suggested to modulate endogenous SL production (35, 36). The endogenous SL metabolism is highly complex. Cers are the branch point of SL homeostasis. They are synthesized *via* SM/cerebroside hydrolysis, *de novo* synthesis (palmitoyl-CoA + serine) and *via* the salvage pathway (e.g. Cer to S1P/C1P and vice versa). Subsequently, many SL metabolites can be generated from the Cer building blocks.

Variations between SMs from different food products, as well as batch-to-batch variations, are important to consider when interpreting study results (37).

Cer and C1P

Cer is a pro-apoptotic molecule. It has been reported to work *via* caspase-dependent and independent mechanisms (24). Additionally, cellular Cer content has been linked to inflammation and metabolic diseases. Cers are synthesized *via* three endogenous pathways (SM/Cerebroside hydrolysis, *de novo* synthesis, salvage pathway). Due to several degradation mechanisms, the level of Cer metabolites is stable. By an increased generation of Cers or by prevention of degradation, an accumulation of Cers may occur provoking excessive apoptosis. Tumor necrosis factor (TNF)-α activates SMases leading to an accumulation of Cers. Elevated TNF-α levels are associated with lipotoxicity by activation of caspases, protein kinase C, serine/threonine protein phosphatase and cathepsin D activity (51). Moreover, promotion of insulin resistance is observed by antagonizing insulin signaling (52). HPLC, GC-MS and MALDI-MS approaches recently revealed insights into the complexity of dietary Cer structures, suggesting a much more complex function of the SL metabolite (53, 54). In accordance with this theory, recent studies reported not only pro- inflammatory effects of Cers. There is increasing evidence that higher Cer content of cells can prevent Lipopolysaccharide- (LPS-) stimulated inflammatory responses (24). Moreover, Cers produced in genetically modified yeast was reported to inhibit TNF-α signaling resulting in stable cell viability (55). Also, orally administered plant derived Cer-precursor SLs have been used as dietary supplements to restore skin barrier function in humans (56).

The Cer metabolite C1P has emerged as a bioactive SL metabolite involved in cell proliferation, macrophage migration, and inflammatory response. It prevents cell death in bone marrow derived macrophages and inhibits activation of caspases. It was further demonstrated to block alkaline SMase and subsequently the formation of Cers, suggesting C1P to antagonize Cer function (57). Mechanisms involving C1P have been described to be mainly located in intracellular compartments. Cer kinase is activated by different agonists, such as IL-1β, macrophage colony stimulating factor or calcium ions. Knock-down of the C1P transfer protein in mice resulted in increased levels of IL-1β and IL-8 levels and enhanced inflammasome assembly (58). A recent investigation has demonstrated a G (i) protein coupled plasma membrane receptor, suggesting C1P as an extracellular ligand to mediate chemotaxis (59). By stimulating phagocytosis in neutrophils and activating degranulation in mast cells, C1P is thought to respond to inflammation (60, 61). C1P reduces TNF-α production by inhibiting its post-translational modification (19, 62). In summary, these data suggest C1P involvement in inflammation as a feedback regulation to Cer stimuli. However, its precise role in inflammation remains to be elucidated.

Sph and S1P

Sph are the most common sphingoid backbones of mammalian SLs. Their presence increases the permeability of phospholipid membranes. Moreover, Sph has been described to act like the structural counterpart of glycerol (63). Of interest, dietary D-erythro-Sph has been suggested to protect the human skin by altering skin microbiota (64). Topical Sph was suggested as effective treatment option for microbial skin diseases (65). It is

TABLE 1 | Summary of SL content in important food sources.

SL metabolite	Dietary Source	mg/100g	References
Sphingomyelin	Bovine milk (whole)	9	(38, 39)
	Cottage cheese	139	
	Buttermilk quark	74	
	Egg yolk	82	
	Beef	44-69	
	Mackerel	224	
	Human breast milk	3-14	
Cerebrosides (total)	Wheat flour	226	(40)
	Soybean	310	
	Barley	275	
	Rice	11.5	
	Corn	11.5	
	Pumpkin	145	
	Cabbage	36.5	
	Spinach	192.9	
	Broccoli	112	
	Sweet potato	67	
	Potato	15.6	
Glucosylceramides	Sugar beet*	12-45	(39, 41)
	Potato*	1-20	
	Apple*	49- 94	
	Wheat*	20	
	Soybean*	20-39	
	Bovine milk	0.7-1.9	
Lactosylceramides	Bovine milk	0.8-1.2	
Gangliosides	Bovine milk	0.5-11	(39, 42, 43)
	Anchovies	9.9	
	Egg yolk	16	
	Chicken (liver)	29	
	Chicken (meat)	0.4–1.5	
	Beef	0.3-0.9	
	Pork	0.5	
	Crab	0.5	
	Squid	0.7	
	Human breast milk	19 - 26 (mg/l)	

**Information was obtained from a study examining dry by-products of the food industry. Information on quantitative content of different SLs is limited and biased by the central research question of the studies. Quantification of the most abundant SLs in dietary sources is pivotal to define nutritional recommendations suitable for balancing SL homeostasis. When evaluating differences in Western versus vegetarian or vegan diets, there is a urgent need of quantitative examining cerebroside composition in food. Content of SM has been reported to vary depending on diet and maintenance of the animals (37).*

still matter of discussion whether the skin barrier-improving effects only depend on the microbiota alteration or Cer synthesis activation in the skin. Current research further aims to evaluate Sph as topical and protective antibiotic (64, 65) and as anti-proliferative agent (63).

S1P derives from ingested SM or cellular membrane SM, which is converted into Sph and phosphorylated to S1P by Sphingosine Kinases (SphKs). SphKs 1 and 2 are highly expressed in lung, small intestine, spleen and stomach (66). Although, most cells are producers of endogenous S1P, the SL is subsequently degraded by S1P lyase, which is found in high levels in tissues such as the small intestine (67). In most tissues S1P levels remain at a baseline. In lymph and blood, S1P levels range from low micromolar to several hundred nanomolar due to the lack of S1P lyase or an enhanced SphK activity (68, 69). Platelet derived growth factor 6 induces Cer production by SM hydrolysis, which is then further metabolized to Sph and S1P. Previously, S1P has been reported to work intrinsically as well as extrinsically. It serves as a second messenger regulating calcium homeostasis in the cell. Its extracellular functions depend on five membrane-bound G-protein coupled receptors (S1P1-5). S1P1 has been reported to contribute to elevated vascular integrity by effecting endothelial adherence junctions. S1P2 and S1P3 are considered to improve vascular contraction. A lack of S1P2 is associated with vascular barrier leakage (70). Recent findings reported a protective effect of enhanced blood S1P levels in allergic mice, suffering from anaphylaxis, as well as a faster recovery after anaphylaxis by enhanced clearance of mast cell mediators (32). In contrast, enhanced tissue S1P levels have been reported to promote inflammation. S1P1-5 are expressed on the surface of several lymphocyte cell types including mast cells and eosinophils (71, 72) suggesting S1P to be crucial for immune cell migration and activation. Especially, S1P1 and S1P2 are important regulators of mast cell and eosinophil responses. Mast cells have been reported to be potent producers of endogenous S1P due to enhanced SphK activation (18). Alterations in S1P homeostasis by deletion of SphK1 or SphK2 were demonstrated to affect sensitization and effector phase in food allergy. Splenocyte analysis by flow cytometry found reduced populations of CD4+ effector T-cells in both SphK knock out strains, as well as a reduced allergy effector cell influx in the gastric mucosa. Enhanced barrier permeability was detected in CaCo2 monolayer stimulated apically with S1P (73). This provides evidence that presence of SphK influences allergen uptake by regulation of the GI barrier integrity. Therefore, S1P effects might depend on a concentration gradient. While enhanced blood S1P levels might be beneficial for barrier integrity, an increase in tissue S1P levels promotes barrier disruption and inflammation. Considering the dietary uptake of SLs, an enhanced need for S1P lyase activity in the small intestine seems logical. However, higher serum S1P levels together with Cer metabolites such as N,N-dimethylsphingosine have been reported to be associated with enhanced anxiety-like behavior in studies investigating associations between SL homeostasis and psychologic disorders and pain syndromes (74, 75). Without any doubt, additional investigations on the metabolite gradient need to be performed.

SLs AND THE GUT WITH FOCUS ON GI BARRIER INTEGRITY

As essential structural components of GI cell membranes, SLs influence barrier integrity and function (**Figure 3**). The GI tract is lined by a single cell layer of different, constantly self-renewing epithelial cell types, termed intestinal epithelial cells (IECs). The luminal side of this single-cell barrier is covered with an alkaline, antimicrobial mucus layer, protecting the barrier from direct contact with the commensal gut microbiota and digested nutritional compounds. On the basolateral side, the GALT is located, which consists of lymphocytes being nourished by blood and lymph vessels and surrounded muscles embedded in loose connective tissue (8).

SLs Influence Cell Differentiation Along the Crypt-Villus Axis

SLs are found throughout the whole GI tract, but preferably located in the apical membrane of IECs (67). Differences in SL distribution have been reported with a higher concentrations of Sph and glucosylceramide in the villi and trihexosylceramide in the crypts (76). This suggests that specific SL metabolite distributions along a crypt-villus axis may stimulate cell differentiation. In support, five of the six Cer synthases have been found in intestinal mucosal cells (33). Recent investigations suggest an imbalance in intestinal SL metabolite distribution to enhance inflammation. Enhanced levels of SM, Cer, S1P and C1P and decreased levels of cerebrosides and gangliosides were found in rodent studies of dextran sulfate sodium colitis (77). Moreover, high levels of SM and Cer have been detected in ilea of Crohn's disease patients (78). TNF-α was demonstrated to upregulate the *de novo* synthesis of Cers in colon cancer. In contrast, inflamed intestinal tissue showed decreased levels of gangliosides, which was reversable by dietary ganglioside supplementation (79). Thus, dietary supplementation of specific SLs might act beneficially in gut inflammation by preserving morphological features important for intestinal function and immune homeostasis (33, 76, 80).

Leaky or Tight? – Interaction of SLs With Cell Junction Related Proteins

It is well known that a disruption of the intestinal cell junctions enhances GI barrier permeability. As a consequence, luminal food and microbial antigens increasingly pass the GI barrier and might interact with GALT resulting in an altered immune response. This "leaky gut" is common in patients with digestive disorders. Absorption of LPS is increased, leading to chronic inflammation, which initiates the onset of metabolic diseases typical for Western societies, such as diabetes and non-alcoholic fatty liver disease (81, 82). Exogenous SMase was previously demonstrated to increase transepithelial permeability. At concentrations as low as 0.01 enzyme units/ml, transepithelial resistance was decreased. The barrier disruption was associated with accumulations of Cers and simultaneously decreased SM and cholesterol levels in membrane fractions containing tight junction proteins occludin and claudin-4 (83). Also, decreased amounts of cholesterol at the plasma membrane resulted in failure of occludin and claudins to localize at the tight junctions (84). A better understanding of the underlying mechanisms of tight junction formation is important to clarify the role of dietary SLs on intestinal barrier integrity.

Combating Inflammation by Changes in Lipid Raft Composition

Lipid rafts are microdomains in plasma membranes rich in SLs and cholesterol, which harbor a variety of signaling and transport proteins. They are specialized in signaling allowing a closer interaction of protein receptors for signal transduction due to kinetically favorable conditions (85). In enterocytes, the brush border is a highly specialized membrane designed to absorb dietary nutrients and to simultaneously form a barrier towards luminal pathogens. Cholesterol or caveolin depletion in membranes was shown to inhibit inflammatory signaling by disrupting microdomain structure. Dietary ganglioside-induced reduction in cholesterol content reduced pro-inflammatory mediators in the intestinal mucosa after acute exposure to

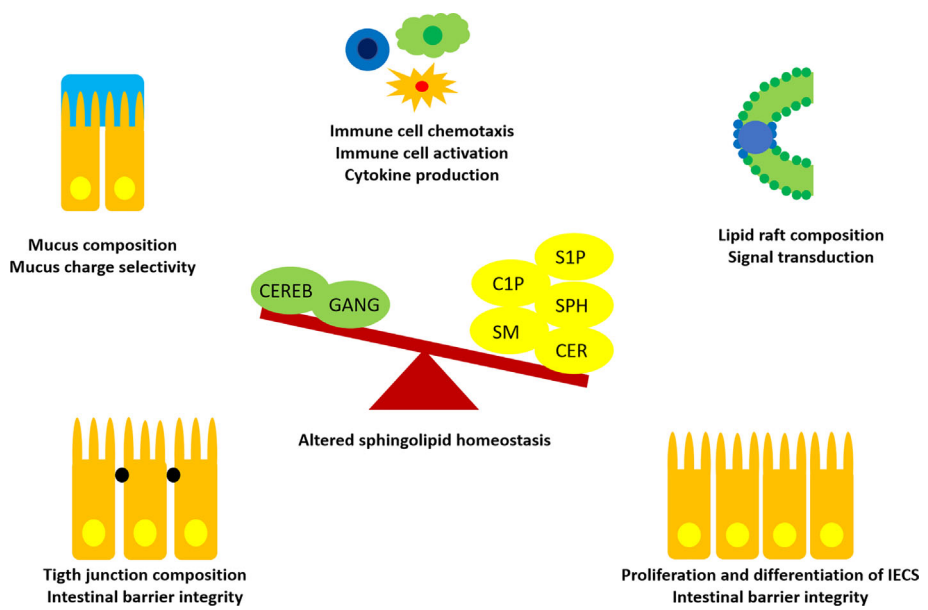

FIGURE 3 | Influences of SLs in intestinal homeostasis. Recent findings suggested an imbalance of SLs and metabolites in favor of S1P, C1P, Cers (CER), Sph, SM, instead of more complex cerebrosides (CEREB) and gangliosides (GANG) to promote intestinal inflammation. SLs are able to interact with IECs and immune cells at different sites. Thus, SLs are promising candidates for treatment of immune-mediated diseases and as predictive biomarkers.

bacterial endotoxin (79). Moreover, a dysregulation in lipid rafts initiated by an accumulation of Cers was found in primary bronchial epithelial cells of cystic fibrosis patients (86). Cholesterol rich lipid rafts were reported to enhance inflammatory activity of TLR4 and 3 agonists. A depletion of cholesterol downregulated inflammatory signaling by TLR4 (87). Thus, effects of dietary SLs on lipid raft formations are promising targets for further studies in inflammatory diseases.

SLs and Their Impact on Mast Cell Activation

Interactions between mast cells and modulating lipids are recently emerging and provide novel insights in underlying mechanism of the gut-brain axis. Mast cells are tissue resident immune cells, often located in skin and mucosa. They are well known for their secretory granules, which degranulate upon mast cell activation and release mediators such as histamine, proteases and cytokines. Mast cells are of high relevance in allergy research, since they respond to allergen exposure with IgE-mediated degranulation leading to tissue damage. Recently, their extensive, uncontrolled degranulation has been associated with an irritable bowel syndrome (IBS) and myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS), both strongly associated with infections or stress situations (88, 89). Mast cell interactions with cells of the central nervous system are considered to link stress with GI symptoms. Furthermore, degranulation of mast cells is calcium-dependent. The entry of calcium is modified by Cers, which can be produced from SM by acid SMase. A rodent study demonstrated exogenously stimulated Cer production to trigger apoptosis of mast cells in acid SMase knock-out mice (31). Thus, exogenously stimulated Cer production might be a powerful tool for dietary supplementation in situations of mast cell activation. Dietary recommendations focusing on mast cell activation have been suggested to combat symptom severity in allergy, IBD, IBS and ME/CFS (89). However, the exact role of dietary SLs in excessive mast cell activation and effects on the gut-brain axis has to be elucidated in further studies.

SLs and the Mucus Barrier

Mucins are a family of heavily glycosylated proteins produced by specialized epithelial cells. They contribute to epithelial barrier function and prevent microbial invasion. In turn, certain members of the gut microbiota (commensals and pathogens) are also able to efficiently break down mucins (90). In line, bacteria competing with pathogens for the degradation of mucins are considered as gatekeepers for a healthy gut and have shown great potential as probiotics (91). Host membranes are protected from bacteria through the secretion of alkaline mucus. However, the effect of charge selectivity changes depending on mucus composition. Interactions with the headgroups of SM or phosphatidylcholine are suggested to alter intestinal mucus function. In accordance, intestinal mucus samples of ulcerative colitis patients demonstrated reduced phosphatidylcholine levels compared with healthy controls (92). Alkaline SMase was shown to inactivate a pro-inflammatory platelet activating factor, thus, being able to counteract intestinal inflammation (93). Therefore, the impact of bacterial, neutral and acid SMases have to be considered when studying SL metabolism in the gut. Data on bronchial mucosal barrier functions are available, due to intensive research on SLs in respiratory diseases. These results might assist investigations on GI mucosal barrier function. Bacterial SMases were found to strongly inhibit transmembrane conductance regulator function in cystic fibrosis reducing mucosal fluidity (94). Analyses of sputum revealed enhanced levels of SM and glycosphingolipids in samples of cystic fibrosis patients (95). A study examining the association of plasma S1P levels with CFTR function and clinical symptom presentation found reduced levels of unbound plasma S1P accompanied by GI symptoms in cystic fibrosis patients (96). Furthermore, enhanced Cer accumulation through neutral SMase activity was monitored in a study investigating the sputum of smokers. The enzyme is activated by TNF-α and interferon-gamma (IFN-gamma) stimuli (97). However, differences in signaling pathways activated by alkaline, neutral, acid or bacterial SMases still remain elusive.

SLs AND MICROBIOTA

Microbial SL Metabolism

While the importance of mammalian SL turnover for mediating various cellular processes is well recognized, mechanisms of microbial SL metabolism are not adequately understood. Prokaryotic SL production has first been described in members of the phylum *Bacteroidetes* (e.g. *Bacteroides, Prevotella, Porphyromonas, Sphingobacterium*) (98) and more recently also in some *Proteobacteria* (e.g. *Sphingomonas, Bdellovibrio, Acetobacter*) (99, 100). Of interest, many of the known bacterial SL producers are also associated with eukaryotic hosts, indicating a close symbiotic relationship. In fact, SL-mediated bacteria-host interactions have been unveiled in a number of plants, animals, as well as unicellular eukaryotes, suggesting an evolutionary early development of this trait (101). The potential influence of bacterial SL production on the host is further underpinned by the abundance of the *Bacteroidetes* phylum, constituting up to 30-40% of the human gut microbiome (101). The gut microbiome represents a highly complex ecosystem with a large potential to influence host health, which has been extensively studied in the last decade. However, the extent of impact of bacterial SLs on eukaryotic physiology, metabolic processes and immune homeostasis is not fully understood yet.

The majority of bacterial SLs are still not characterized and recent studies just begun to uncover the diversity of bacterial SL structures. For example, *Alistipes* and *Odoribacter* species have been found to be responsible for sulfonolipid production in mouse cecum, which correlated with a high-fat diet (102). Additionally, *Bacteroides fragilis* has been shown to produce three different types of SLs, namely, the Cer phosphorylethanolamine, its corresponding dihydroceramide base, and the glycosphingolipid α- galactosylceramide (103). These and other bacterial-derived SLs can pass the epithelial barrier in the gut and enter host metabolic pathways, [as shown by Johnson et al., 2020 (104)]

They administered a SL-producing *Bacteroides thetaiotamicron* to mice and observed increased levels of Cers in liver and reduced *de novo* SL production, which was not achieved when the *B. thetaiotamicron* capability to produce SLs was knocked out (104). In another study mono-colonization of germ-free mice with a SL-deficient *B. thetaiotaomicron* strain led to intestinal inflammation and shifts in SL levels in the intestine (105). Moreover, IBD patients have decreased *Bacteroides*-derived SLs but increased host SLs, further highlighting the role of bacteria-derived SLs in intestinal immune homeostasis (106). Likewise, Duan et al. showed that germ-free mice have a reduced SM hydrolysis capability, suggesting that the intestinal microbiota contributes to SL turnover (107). Thus, microbial SLs have the potential to mediate signaling pathways and influence their hosts lipid metabolism (108). However, the exact mechanisms underlying SL- mediated host-microbial interactions and their implication in diseases warrant further research.

Diet is a key factor shaping the gut microbiome (109). In turn, the gut microbiome also determines glycemic responses to certain foods, which show a high interpersonal variability based on individual microbiome features (110). Thus, it is imperative to unravel the basic metabolic processes and dynamic interactions of microorganisms that are linked to nutrition and diet. Since dietary SLs are essential components of eukaryotic cellular membranes, they can be found in virtually any type of food. Yet, our knowledge about the capability of microorganisms to degrade dietary SLs is limited. Just recently, first evidence on the microbial assimilation of dietary sphinganine in the mouse gut has been established by using a click-chemistry based approach (termed ClickSSS) to track the incorporation of bio-orthogonal dietary omega-alkynyl sphinganine into the gut microbial community (111). Bacteria from the *Bacteroides* genus were almost exclusively involved in the assimilation of sphinganine, although other non-SL-producing bacteria (e.g. *Bifidobacterium, Lactobacillus*, and *Turicibacter*) have been discerned to have a role in SL metabolism as well. In the future this and similar approaches should be used to identify yet uncharacterized microbial processes implicated in SL metabolism. Investigating the influence of dietary SLs on the gut microbiota is key to understand the tight connections between SL metabolism,the gut microbiota, and host immune homeostasis.

SL-Mediated Host-Pathogen Interactions

IECs and other intestinal cells are in permanent cross-talk with the microbiome and with bioactive compounds. It is supposed that SLs, e.g. SM, have protective effects at the mucosal site, thus being able to prevent the invasion of pathogenic microorganisms (20). SM is also taken up by enterocytes, which subsequently degrade the lipid backbone for reutilization (112). Degradation products of SLs and glycosphingolipids also interact with the immune system of the host (**Figure 4**). Sph and lyso-SLs, both derivatives from SL degradation, have antimicrobial properties against gram-positive and gram-negative pathogenic bacteria (e.g. *Pseudomonas aeruginosa, Staphylococcus aureus, Acinetobacter baumannii, Campylobacter jejuni, Listeria monocytogenes* and *Clostridium perfringens*), as shown in *in vitro* and *in vivo* experiments (20, 113, 114). Multiple epithelial tissues benefit from the preventive effect of Sph on severe pathogen infections involving innate and immediate defense mechanisms (113). The high expression of Sph in human nasal epithelial cells is associated with protective barrier effects, and decreasing Sph levels promote bacterial infections (115). Bacterial survival fluctuations during mouse lung infection were experimentally modified by deletion of a microbial sphingosine-responsive transcription factor (sphR), suggesting that sphR of pathogens plays an important role in the initial response to host infection (116). Also S1P has effects on infection dynamics *via* immune cell trafficking and differentiation as well as preserved barrier integrity (117, 118).

Glycosphingolipids act as antigens, as receptors for microbial products and toxins, and as mediators for cell adhesion in eukaryotic cells (119–121). They are suggested to have a functional role in host immune responses and for pathogen's escaping strategies (122). Lactosylceramide (LacCer), a glycosphingolipid including Cer and lactose, has a special role in recognizing pathogen-associated molecular patterns. It activates phagocyte function (123, 124) and there is evidence for direct binding to bacterial pathogens (shown for *Escherichia coli, Bordetella pertussis, Bacillus dysenteriae, Propionibacterium freudenreichii*) and fungi (shown for *Candida albicans*) (125–130). Increasing cellular Cer accumulation was found to parallel production of antimicrobial peptides (AMPs), which are key components of antimicrobial barrier functionality and innate immunity (131). C1P has been reported to directly activate cytosolic phospholipase A2 which subsequently leads to production of the AMPs human beta-defensin 2 and human beta-defensin 3 (132) Increased S1P levels were reported to strongly stimulate the expression of cathelicidin antimicrobial peptide when the epidermis is under stress, i.e. in response to attack of microbial pathogens (133). At the host neutrophils plasma membrane, LacCer accounts for 70% of glycosphingolipids, indicating its importance in pathogen binding (122, 134), and endogenous LacCer supplementation to neutrophil cell lines with low levels of LacCer can rescue their phagocytic activity (123).

SL HOMEOSTASIS

The intense investigations on SLs in the last years revealed SL metabolism as a universal response to stress. Depending on the source and type of stress, different signaling pathways are activated, which provides explanations for the complex and often enigmatic nature of SLs. Recent innovations in systems biology, big data analysis, genomics and epigenetics enabled detailed analyses of SL structures resulting in highly interesting novel findings on the role of SLs in inflammation, disease progress and nutrition.

SLs and the Drug Industry

The most intense studied bioactive SLs include Cer, Sph and S1P (135). The Sph analogue ISP1 (Myriocin) showed beneficial effects in treating insulin resistance and the metabolic syndrome (136, 137). Short-chain analogues of Cers are of

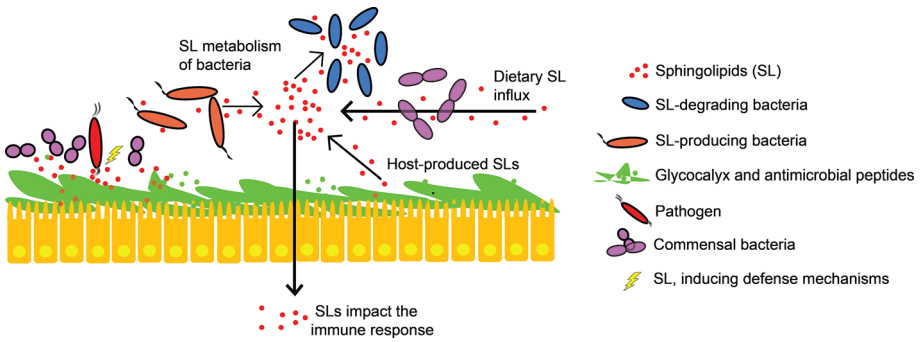

FIGURE 4 | Sphingolipids in host-microbial interactions. Epithelial cells that produce various antimicrobial peptides and secrete mucin (glycocalyx) prevent microbial invasion. SLs alter intestinal mucus function, compete with commensals for epithelial binding sites and induce defense mechanisms against pathogenic bacteria. Intestinal SL levels are not only influenced by diet and endogenous SL-production, but also by SL-producing and SL-degrading bacteria. Bacterial and host SLs are structurally similar. They mediate specific host immune responses and interact with signaling pathways.

special interest in the treatment of leukemia (138). Especially, S1P has been in the focus of many recent studies, as its two kinases (SphK 1 and 2) and its five G- protein coupled receptors (S1P1-5) received increased attention in research as therapeutic targets. Effects were shown in treatment approaches of diseases common for Western societies, for instance, ulcerative colitis (139), allergy (32), multiple sclerosis (140) and cancer (141). Furthermore, the SL metabolite is suspected to be involved in the onset and progression of psychiatric disorders and pain syndrome (75) which might provide evidence to find a missing link between GI inflammation and psychosomatic manifestations. The S1P analogue FTY720 (Fingolimod) was originally used in treatment of multiple sclerosis by modulating immune cell chemotaxis, but has been also suggested for cancer treatment (142) and for restoring endothelial barrier dysfunction (143).

Recent analyses focusing on the SL metabolism suggest C1P, cerebrosides (especially glucosylceramide and lactosylceramide) and gangliosides as novel emerging candidates for therapeutically targeting cancerogenic, immune-related and inflammatory disorders. Enzymes, especially SMases and ceramidases are in the center of recent investigations. Neutral SMase inhibitors show an enormous potential for treatment of inflammation in cardiovascular, pulmonary and neurological systems (144). Alkaline SMase is a promising target in GI disorders, due to its high activity in the digestive system. It has been suggested to regulate mucosal growth in an alkaline SMase knock-out mice model (145) and to reduce gut inflammation (93) in a rodent study. Acid SMase has been reported to reduce severity of cystic fibrosis (115), acute lung injury (146) and Wilson disease (147). When evaluating acid SMase as drug target, it is essential to mention, that the enzyme seems to work in a compartment specific manner. Thus, specific roles of acid SMases have to be further elucidated (148). Ceramidases have been suggested as promising treatment targets in many diseases. Inhibitors of acid ceramidase and neutral ceramidase overcome cell death resistance after prolonged anti-cancer treatments. Essential work is yet to be done to define inhibitors suitable for combining specificity and ability to reach specific cellular compartments (144). Although, promising candidates have been identified, their exact indication needs to be further investigated.

To date it remains unclear how to restore the SL balance in situations of altered SL homeostasis. However, there is strong evidence suggesting dietary SLs to be suitable candidates. Cers are well known targets when talking about skin barrier function. Reduced levels have been reported in situations of barrier dysfunction, as seen in psoriasis (149) and atopic dermatitis (150). Previously, the effect of supplementation of synthetic and animal-based Cer-precursor-SLs have been investigated. However, with the discovery of an SL efflux mechanism of rodent enterocytes (44) and the possibility to isolate the SLs from conventional plant-based food products, which diminished concerns about infectious diseases disseminating *via* animal SLs (151), plant SLs arespeculated to be safer. Beneficial effects of oral supplements have been indicated in studies on skin hydration and skin barrier reinforcement (56). A high abundance of gangliosides in human breast milk indicates them as beneficial dietary supplements in infant formula, due to their effects on GI inflammatory disorders and neurodevelopment of children (152–154). Altogether, there is increasing evidence supporting safety of dietary SL supplementation to counteract a dysregulated immune homeostasis. Nonetheless, information on differences regarding the source of the Cers (animal, plant, synthetic), structural differences of Cers and subsequent effects are not well studied to date.

SL Metabolites - Novel Biomarkers for Stress Responses?

A dysfunction in SL homeostasis due to reoccurring stress stimuli might provide an explanation for many inflammatory and immune-related diseases of yet unknown origin. To support this theory SL metabolites have been suggested as novel biomarkers for diseases such as Alzheimer's disease and metabolic diseases related to insulin resistance (155, 156). Cers have been identified as cholesterol-independent biomarkers for

familial coronary artery disease by an unbiased machine learning approach, revealing 30 out of 32 Cer types being significantly elevated in sera of patients compared to healthy controls (157).

Establishing a biomarker system suitable for clinics depends on reliable measurements to distinguish health from disease. Several rodent and human studies found enhanced levels of SM, Cers, S1P and C1P associated with IBD and simultaneously reduced levels of glucosylceramides and monosialodihexosylgangliosides (77, 78, 158). A reduction of alkaline SMase activity was suggested to be a potential trigger for the dysregulation in SL metabolism as seen in a study of human chronic colitis (159). In a human clinical study, a high throughput whole exome sequencing approach was used to identify mutations in a chronic kidney disease and sensorineural hearing loss patient including also family members (160). This enabled the identification of a gene defect in RMND1, which leads to an accumulation of Cer and subsequently promote dysregulated apoptosis and tissue necrosis in kidneys. A broader accessibility to more advanced chemical analysis techniques such as UHPLC-High resolution mass spectrometry allows identification of SL species as biomarkers of clinical significance (161).Thus, not only methodological innovations will support sphingolipidomics, but also the dissemination of knowledge on accurate mass, isotopic patterns, and collision-induced fragmentation together with enlarged compound libraries suitable for identifying a broader spectrum of SL species is essential.

CONCLUSION

SLs are important constituents of cell membranes and enable a fast and efficient transduction of cellular signals. Their highly complex metabolism is strictly regulated. Our diet has been demonstrated to influence SL homeostasis and to shape the gut microbial community. With regards to a balanced SL metabolisms, it is paramount to clarify the role of microbial SL producers and microbial SL metabolites. Western diets are hypothesized to dysregulate SL homeostasis and thereby cause an increase in prevalence of cancerogenic, immune-related and inflammatory diseases. In this context, immuno-nutrition can be a powerful tool to counteract chronic inflammation, overall morbidity, and increasing health care costs. Thus, it is imperative to further elucidate the SL metabolism and to define dietary recommendations in order to restore a dysregulated SL homeostasis. Furthermore, the impact of bacterial SLs on eukaryotic physiology, metabolic processes and immune homeostasis need to be in focus of future studies evaluating the role of SLs in immuno-nutrition. Recent technical innovations in system biology, genomics and epigenetics pave the way for such complex, holistic analysis of SLs. Additionally, unbiased machine learning approaches might constitute a key tool in upstream analysis of multidisciplinary data sets deriving from metabolomics, microbiome analysis and clinical studies. Although, many questions remain to be answered, it is clear that a detailed insight in the highly complex nature of SL homeostasis is pivotal to combat chronic, low-grade intestinal inflammation and subsequent metabolic diseases within the human body.

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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Modulation of Immune Responses by Nutritional Ligands of Aryl Hydrocarbon Receptor

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Accumulating evidence indicates that nutrition can modulate the immune system through metabolites, either produced by host digestion or by microbiota metabolism. In this review, we focus on dietary metabolites that are agonists of the Aryl hydrocarbon Receptor (AhR). AhR is a ligand-activated transcription factor, initially characterized for its interaction with xenobiotic pollutants. Numerous studies have shown that AhR also recognizes indoles and tryptophan catabolites originating from dietary compounds and commensal bacteria. Here, we review recent work employing diet manipulation to address the impact of nutritional AhR agonists on immune responses, both locally in the intestine and at distant sites. In particular, we examine the physiological role of these metabolites in immune cell development and functions (including T lymphocytes, innate-like lymphoid cells, and mononuclear phagocytes) and their effect in inflammatory disorders.

Keywords: aryl hydrocarbon receptor, AhR, intestine, microbiota, tryptophan, immunity

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INTRODUCTION

Food represents not only a source of nutrients for the maintenance of essential biological functions, but also contains dietary components that regulate immune cell populations. These include microbiota-derived short-chain fatty acids, polyamines, and indoles derivatives, which are ligands of the Aryl Hydrocarbon Receptor (AhR) (1).

AhR is a ligand-activated transcription factor residing in the cytosol. Upon binding to an agonist, AhR translocates to the nucleus where it forms an active heterodimer with ARNT and promotes the transcription of genes that are under its control. AhR is expressed in multiple immune cells such as myeloid cells, innate lymphoid cells, B lymphocytes and certain subtypes of T cells. AhR activation has an overall anti-inflammatory and immunoregulatory role in innate and adaptative immunity, both in steady-state or in inflammatory scenarios such as autoimmunity or infection (2). However, a number of these observations were made *in vitro* or *in vivo* by injecting AhR agonists in non-physiological routes or concentrations. Some studies have also used AhR ligands of xenobiotic origin, which are known to induce aberrant AhR signaling (3). This has led to some contradictory findings, in particular in T cell biology (4).

In this review, we examine the physiological role of nutritional AhR ligands in immune cells and immune responses by focusing on experimental results obtained by direct intestinal exposure or diet manipulation.

WHAT ARE THE NUTRITIONAL AhR LIGANDS?

AhR was initially described as a receptor for xenobiotic pollutants, mostly aromatic hydrocarbons. However, over the years, physiological ligands have been identified. There are other exhaustive reviews of AhR agonists (5–7), here we focus on nutritional AhR ligands (**Figure 1** and **Table 1**).

AhR ligands are present in the diet, as natural compounds in food. Flavonoids derived from fruits and vegetables such as quercertin and resveratrol have been identified as AhR ligands based on *in vitro* assays, but their physiological relevance is unclear (8–10). The main class of dietary AhR ligands is indoles, including Indole-3-acetonitrile, Indole-3-carbinole (I3C), 3,3'-diindolylmethane (DIM) and Indolo(3,4)bicarbazole, which are found mainly in cruciferous vegetables like broccoli or Brussel sprouts (11). After consumption, I3C is converted in the stomach by acid-mediated condensation into various byproducts, including high affinity AhR ligands DIM and indole[3,2-b] carbazole (ICZ) (11).

Another source of nutritional AhR ligands is microbiota metabolism, particularly tryptophan catabolism. Some species of bacteria such as *Lactobacillus* can use tryptophan instead of glucose as a source of energy, and produce AhR ligands such as indole-3-acetic acid (IAA), tryptamine (TA) and 3-methyl indole (14, 15). A well-described example is *Lactobacillus reuteri*, producing the AhR ligand indole-3-aldehyde (IAld) (15). Moreover, Tryptophanase-expressing bacteria, which are mostly ampicillin-sensitive and vancomycin-resistant, degrade tryptophan into indole that is further metabolized by host liver cells into AhR ligands, such as indoxyl-3-sulfate (I3S) and indole-3-propionic acid (IPA) (16). In addition, it has been reported that butyrate and other short-chain fatty acids (SCFA), which originate from the fermentation of dietary fibers by the microbiota, can activate AhR signaling in reporter cell lines (12). Whether this observation holds true in other cell types remains to be confirmed. In particular, another study failed to detect AhR activation in B cells upon *in vitro* exposure to butyrate, while 5-hydroxyindole-3-acetic acid (5-HIAA), a serotonin metabolite, activated AhR signaling in B cells *in vitro* and *in vivo* (13).

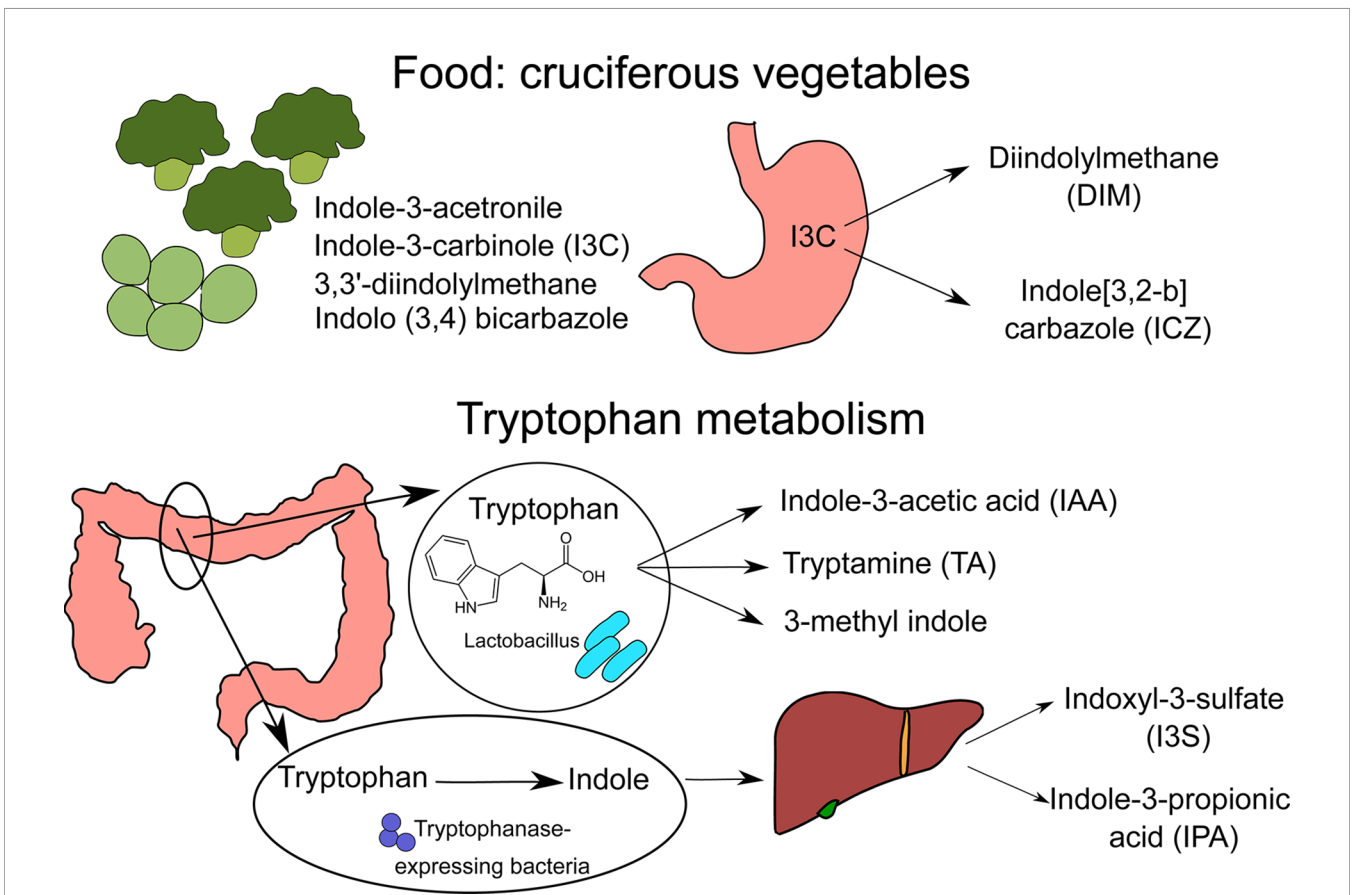

FIGURE 1 | Sources of nutritional AhR ligands. Nutritional AhR ligands are derived either from the breakdown of food components or from tryptophan catabolism by intestinal microbiota. Several types of indoles are present in cruciferous vegetables. In particular, indole-3-carbinol (I3C) is converted in the stomach into high affinity AhR ligands Diindolylmethane (DIM) and indole[3,2-b]carbazole (ICZ). Tryptophan is metabolized by *Lactobacillus* bacteria into indole-3-acetic acid (IAA), tryptamine (TA) and 3-methyl indole. In addition, tryptophanase-expressing bacteria degrade tryptophan into indole, which is metabolized by host liver cells into indoxyl-3-sulfate (I3S) and indole-3-proprionic acid (IPA).

TABLE 1 | Nutritional AhR ligands classification.

Compound	Abbrevations	Source	Reference
Quercertin		Dietary ligands	(8–10)
Resveratrol			
Indole-3-acetonitrile	IAN		(11)
Indolo(3,4)bicarbazole			
Indole-3-carbinole	I3C		
3,3'-diindolylmethane	DIM	Host metabolism from food components	
Indole[3,2-b] carbazole			
Linear trimer, [2-(indol-3-ylmethyl)-indol-3-yl]indol-3-ylmethane	LTR_1		(12)
1-(3-hydroxymethyl)-indolyl-3-indolylmethane	HI-IM		
5-hydroxyindole-3-acetic acid	5-HIAA		(13)
Indole-3-acetic acid	IAA	Microbiota metabolism from food components	(14, 15)
Tryptamine	TA		
3-methyl indole			
Indole-3-aldehyde	IAld		(15)
Butyrate			(12)
Indoxyl-3-sulfate	I3S	Host metabolism from tryptophan catabolites from microbiota	(16)
Indole-3-propionic acid	IPA		

Reported nutritional AhR agonists classified based on their origin.

WHAT IS THE BIODISTRIBUTION OF NUTRITIONAL AhR LIGANDS?

Dietary AhR ligands are released locally in the digestive track but also distributed to other sites *via* the blood. After oral administration, the biodistribution of I3C and its acid condensation products has been analyzed by high-performance liquid chromatography. I3C is absorbed from the gut and distributed systemically into a number of well-perfused tissues (17). I3C level peaks at 15min and decreases considerably 1h after administration in the plasma, liver, kidney, lung, heart and brain. However, I3C products DIM, LTR_1 and HI-IM are detectable in these organs from 15 min and persist after 6h, and up to 24h in the liver. ICZ, another product from I3C, was also identified in the liver, but not in plasma or other organs, at 6h and 24h after I3C administration (17). In line with those observations, I3C was detected in serum 15 min after gavage, but not when administered as supplement in the chow diet. By contrast, DIM was detectable in the serum of mice fed with I3C-supplemented chow diet (18). In a study carried out in women given a single oral I3C dose, DIM, but not I3C itself, was detected in plasma peaking at 2h and returning to basal levels 24h after administration (19). These observations suggest that I3C is rapidly cleared from the circulation, while its condensation products can reach distant organs and exert longer lasting effects.

It has also been proposed that nutritional AhR ligands can cross the blood-brain barrier. After intra-peritoneal injection, I3S was detected in the brain (20), however oral administration was not examined.

Finally, microbiota-derived AhR ligands have been detected in breastmilk. After gavage of pregnant mice with radiolabeled *E.Coli*, a species which expresses Tryptophanase, labelled AhR ligands were detected in maternal milk, including indole-3-lactic acid, showing the transfer from maternal intestinal microbiota to milk (21).

DO NUTRITIONAL AhR LIGANDS INFLUENCE MICROBIOTA COMPOSITION?

Tryptophan content in the diet shapes significantly the microbiota composition. Tryptophan supply relies exclusively on the diet since the host cannot synthetize it. After 2 or 3 weeks of dietary intervention, mice fed with a tryptophan-deprived diet display an increase in fecal *Actinobacteria* and *Proteobacteria*, and lower relative abundance of *Bacteroidetes* and bacteria belonging to *Firmicutes* phylum such as *Lactobacillus and Staphylococcus* (22, 23). In addition, after 4 weeks of tryptophan-low diet, the abundance of *Lactobacillus reuteri* is also decreased in the stomach (15). Of note, tryptophan is metabolized in the gut not only into AhR ligands by microbiota, but also through the serotonin and kynurenine pathways by host cells (5, 24). Whether the observed alterations in microbiota composition are entirely dependent on changes in AhR ligands availability remains to be confirmed. In addition, caution should be exercised when interpreting *in vivo* experiments employing tryptophan-low diets, as modification in microbiota diversity by itself may impact the outcome.

By contrast, I3C content in the diet only causes a relatively minor change in intestinal microbiota composition. Normal chow contains phytochemicals that can act as precursors of AhR ligands. Switching from normal chow to a synthetic diet alters fecal microbiota diversity (25, 26). Mice fed with a AhR ligand-free synthetic diet show a decreased abundance in fecal *Bacteroidetes* and increased abundance in *Actinobacteria* and *Firmicutes* compared to mice fed with the same diet supplemented with I3C (25, 26). Relative change observed in *Erysipelotrichaceae* is inconsistent between studies (25, 26). Importantly, most of these alterations in fecal microbiome composition were also observed in AhR-deficient mice,

showing that this occurs independently of AhR signaling (26). In a study employing a synthetic diet supplemented with DIM, no difference were observed in fecal microbiota between groups (27). However, mice fed with the synthetic diet had increased *Bacteroidetes* and decreased *Firmicutes* abundance in the small intestine compared to mice fed with the DIM-supplemented synthetic diet. These differences in microbiota diversity induced by DIM supplementation were abrogated in AhR-deficient mice (27).

These results highlight a complex interplay between microbiota composition and the supply of dietary AhR ligands, with tryptophan having a more pronounced effect than single AhR ligands.

WHAT IS THE IMPACT OF NUTRITIONAL AhR LIGANDS ON INTESTINAL IMMUNITY?

Nutritional AhR ligands are essential for the maintenance of intestinal intraepithelial lymphocytes (IELs) and type 3 innate lymphoid cells (ILC3).

Small intestine IELs are a specialized population of T cells composed of several subsets (TCRγδ, $CD4^{-}CD8\alpha\alpha^{+}$ TCRαβ and $CD4^{+}CD8\alpha\alpha^{+}$ TCRαβ). Mice fed with a AhR ligand-free synthetic diet have lower numbers of TCRγδ and $CD4^{-}CD8\alpha\alpha^{+}$ TCRαβ IELs compared to mice on I3C-supplemented synthetic diet (28, 29). However, these IELs can develop normally in AhR-deficient, indicating that dietary AhR ligands are required for their maintenance rather than differentiation (29). In addition, $CD4^{+}CD8\alpha\alpha^{+}$ TCRαβ IELs are decreased in the intestine of mice fed with a tryptophan-low synthetic diet compared to standard diet and tryptophan-high diet (30). *Lactobacillus reuteri* was identified as essential for $CD4^{+}CD8\alpha\alpha^{+}$ TCRαβ IELs development. However, a tryptophan-high diet in conjunction with *L.reuteri* colonization was not sufficient to induce these IELs in germ-free mice, suggesting the participation of additional factors (30).

ILC3 produce lymphotoxin, controlling the development of intestinal lymphoid follicles. Mice fed with a AhR ligand-free synthetic diet display decreased ILC3 and intestinal lymphoid follicles numbers compared to mice fed on I3C-supplemented synthetic diet (28, 31). Of note, I3C supplementation had no impact when given to AhR-deficient mice (31). In addition, development of ILC3 and intestinal lymphoid follicles are normal in germ-free mice (32), suggesting that dietary AhR ligands, produced independently of microbiota metabolism, are sufficient for ILC3 differentiation or maintenance. ILC3 are the main producers of intestinal IL22, which is critical for the secretion of antimicrobial peptides by intestinal epithelial cells and the defense against intestinal infections (33). Consistent with decreased ILC3 numbers, mice fed on a AhR ligand-free synthetic diet express lower levels in the intestine of antimicrobial peptides such as C-type lectin regenerating islet-derived protein 3 (RegIII) (29, 34), and are more susceptible to infections with *Citrobacter Rodentium* (35) or *Clostridium difficile* (28).

AhR has been proposed to control Treg differentiation. Tregs in the gut express higher levels of AhR than other Tregs in the body (36). AhR expression is necessary for Treg gut homing and function, and is independent of microbiota, since it is not affected in antibiotics-treated mice or germ-free mice. Whether nutritional AhR ligands play a role is unclear, as feeding mice with an AhR ligand-free synthetic diet was reported to be inconclusive regarding Treg development (36).

Lack of dietary AhR ligands worsens the symptoms of intestinal inflammation. In the model of DSS-induced colitis, mice on a synthetic AhR ligand-free diet show more severe symptoms, such as weight loss and tissue damage, compared to mice fed with a synthetic diet supplemented with I3C (26, 29) or with tryptophan (34). Tryptophan supplementation did not modify the severity of symptoms in AhR-deficient mice, confirming the dependency on AhR activation (34). Consistent with these observations, symptoms of DSS-induced colitis were mildly ameliorated when mice fed on normal chow were given tryptophan supplementation in the drinking water (37) or I3C by oral gavage (38). In mice fed on AhR ligand-free synthetic diet, increased epithelial damage may be due to the lack of IL22 production in response to DSS-induced inflammation (26, 34). Importantly, AhR activation in intestinal epithelial cells is also involved in barrier repair during colitis (39).

Finally, in a model of oral tolerance to ovalbumin, mice fed with I3C-enriched chow diet have lower levels of serum anti-ovalbumin IgG1 antibodies, indicating better induction of oral tolerance (18). This was correlated with increased expression in the small intestine of retinaldehyde dehydrogenase, a molecule known to promote Treg differentiation, but the target cells of dietary AhR ligands in this model remain unclear.

Collectively, these observations show an essential role for nutritional AhR ligands in maintaining intestinal lymphoid populations and homeostasis (**Figure 2**).

WHAT IS THE IMPACT OF NUTRITIONAL AhR LIGANDS ON IMMUNE RESPONSES AT DISTANT SITES?

Nutritional AhR ligands can also modulate the differentiation of immune cells outside of the intestinal mucosa, as shown for monocytes. Monocytes circulate in the blood and are recruited to tissues where they differentiate into dendritic cells or macrophages. In mice fed with a synthetic AhR ligand-free diet, monocyte differentiation into dendritic cells is reduced in the skin, compared to mice on an I3C-supplemented diet (40). This is consistent with *in vitro* observations that AhR activation skews monocyte differentiation from macrophages to dendritic cells by controlling the expression of the transcription factors Irf4 and Blimp-1. In addition, monocyte differentiation into dendritic cells in the peritoneum is impaired in antibiotics-treated mice and could be restored by I3C diet supplementation

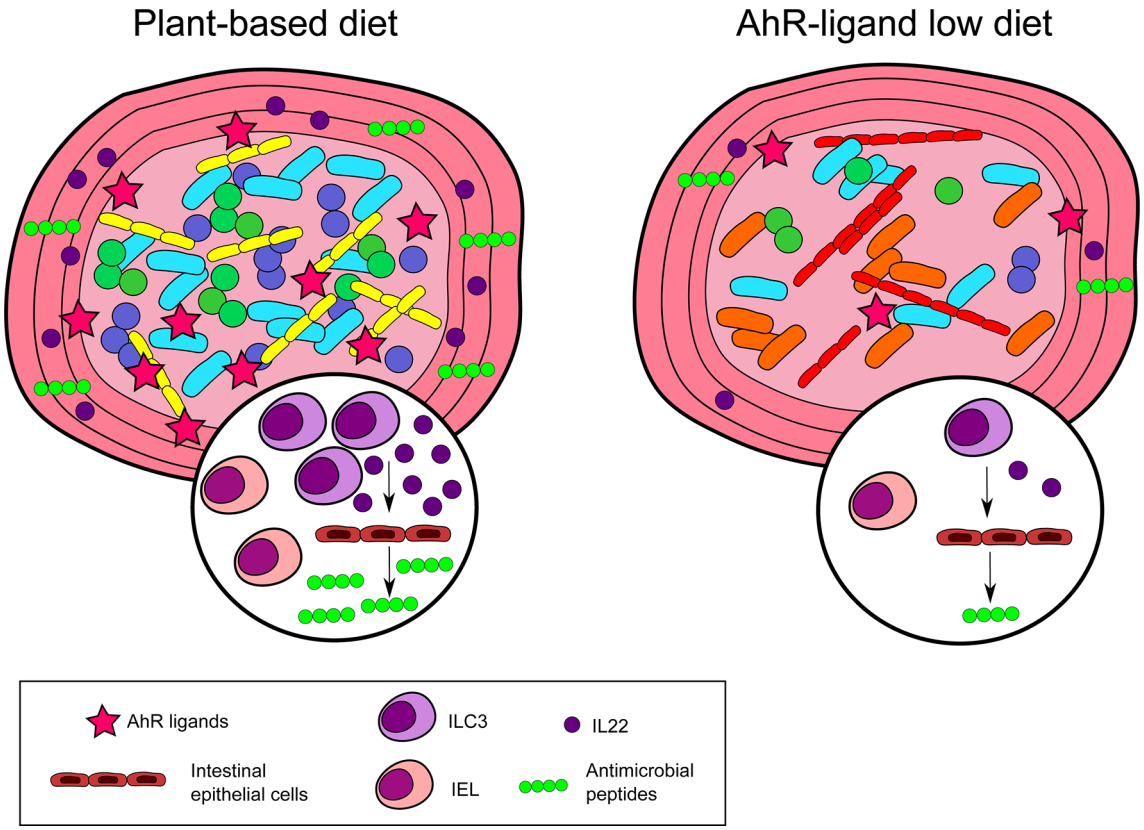

FIGURE 2 | Nutritional AhR ligands in intestinal immunity. Nutritional AhR ligands are involved in the maintenance of intestinal intra-epithelial lymphocytes (IEL) and type innate-like lymphoid cells (ILC3). ILC3 are the main producers of IL22, which acts on intestinal epithelial cells to induce the secretion of antimicrobial peptides. In the absence of dietary AhR ligands, IEL and ILC3 are reduced, and microbiota diversity is altered.

(40), suggesting a role for microbiota-derived AhR ligands in physiological conditions (**Figure 3**).

Deficit in dietary AhR ligands increases the severity of inflammation in the central nervous system, as evidenced in the experimental autoimmune encephalomyelitis (EAE) model. After induction of EAE, mice placed on a synthetic tryptophan-free diet display worse disease scores and delayed recovery compared to mice fed with tryptophan-supplemented diet (20, 41). This difference was abolished in mice deficient for AhR specifically in astrocytes or in microglia, showing that dietary AhR ligands can exert their effect on brain-resident populations. Lack of dietary tryptophan results in the increased expression of pro-inflammatory molecules in the brain such as *Ccl2, Nos2* and *Tnfa* (20, 41). Treatment with ampicillin also delays disease recovery and increases *Ccl2* and *Nos2* expression, which could be reverted by diet supplementation with IPA, IAld, indole or Tryptophanase (20), suggesting a major role in this phenomenon for microbiota-derived AhR ligands.

AhR ligands from microbiota metabolism also influence the differentiation of IL-10 producing regulatory B cells, which are found in lymphoid organs. Gavage with 5-HIAA increases the expression of *Il10* in spleen B cells and reduces the severity of joint swelling in a model of antigen-induced arthritis (13). This effect is abolished in mice deficient for AhR in B cells, confirming the role of AhR signaling.

These observations indicate that AhR ligands participate in the communication between gut, microbiota and distant tissues such as brain and skin.

CONCLUSION AND PERSPECTIVES

There is accumulating evidence that nutritional AhR ligands play an essential role in the maintenance of intestinal immune homeostasis and the control of intestinal inflammation. Several studies also suggest a similar role in distant tissues such as skin and brain. Whether dietary AhR ligands impact other organs or mucosal sites remains to be investigated.

Circadian rhythms regulate some essential aspects of immune activity, such as leukocyte trafficking or inflammatory cytokine secretion (42). AhR has been reported to interact with circadian clock proteins and suppress their transcriptional activity (43). In addition, the supply of nutritional AhR ligands fluctuates across time with feeding behavior, as does microbiota mass and the release of nutritional metabolites (44). It will be important to

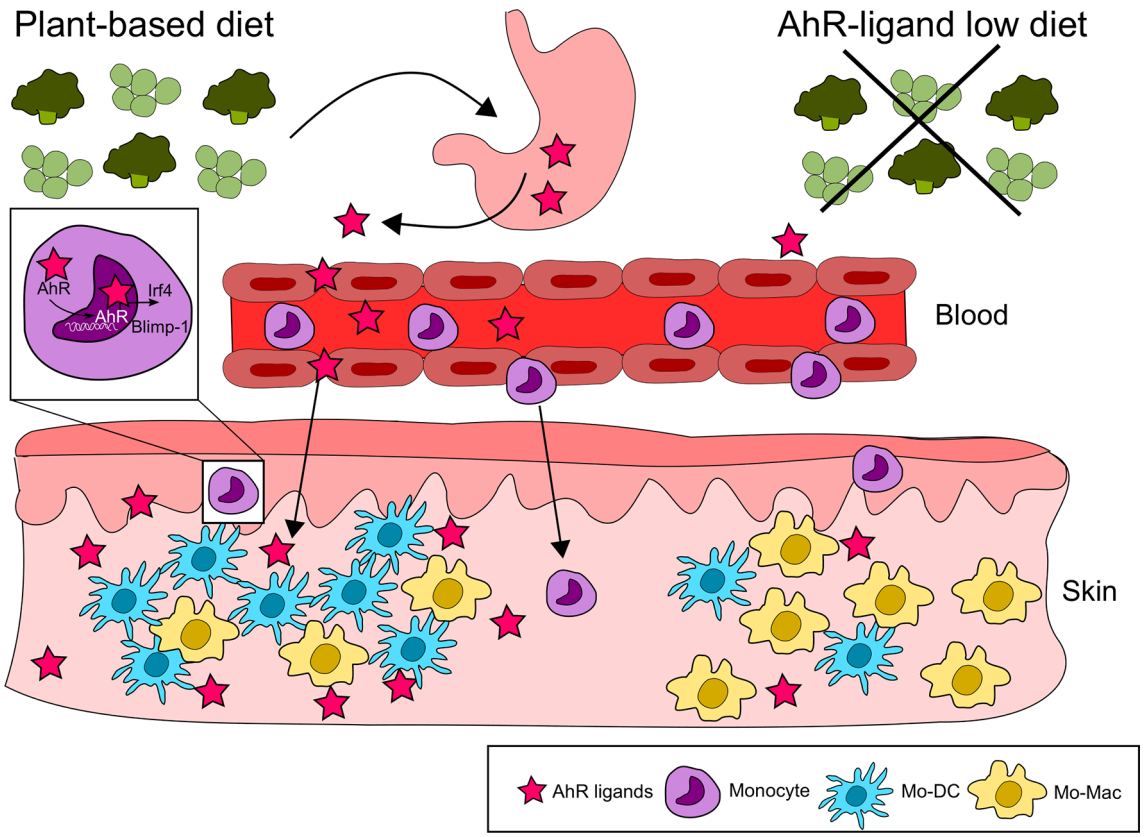

FIGURE 3 | Nutritional AhR ligands in monocyte differentiation. Nutritional AhR ligands modulate monocyte differentiation in the skin. AhR activation from dietary agonists favors monocyte differentiation towards dendritic cells *via* the induction of the transcription factors Irf4 and Blimp-1. In the absence of AhR signaling, monocytes differentiate preferentially into macrophages.

decipher the possible connection between AhR, circadian rhythms and immune cells.

Impaired production of AhR ligands has been observed in the intestinal microbiota of patients suffering from inflammatory bowel disease (45) and celiac disease (22). Given the critical impact of dietary AhR ligands on ILC3 numbers and the maintenance of barrier integrity, diet supplementation with AhR ligands, or AhR ligand-producing bacteria, is an attractive strategy to improve the treatment of inflammatory gastrointestinal diseases (24). However, a better understanding of the role of nutritional AhR ligands on the immune homeostasis of distant tissues and on myeloid cells will be essential to optimize these therapeutic approaches.

AUTHOR CONTRIBUTIONS

AJ and ES wrote the manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Nutrition

REVIEW
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Low-Dose Lactulose as a Prebiotic for Improved Gut Health and Enhanced Mineral Absorption

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Although medium and high doses of lactulose are used routinely for the treatment of constipation and hepatic encephalopathy, respectively, a wealth of evidence demonstrates that, at low doses, lactulose can also be used as a prebiotic to stimulate the growth of health-promoting bacteria in the gastrointestinal tract. Indeed, multiple preclinical and clinical studies have shown that low doses of lactulose enhance the proliferation of health-promoting gut bacteria (e.g., *Bifidobacterium* and *Lactobacillus* spp.) and increase the production of beneficial metabolites [e.g., short-chain fatty acids (SCFAs)], while inhibiting the growth of potentially pathogenic bacteria (e.g., certain clostridia). SCFAs produced upon microbial fermentation of lactulose, the most abundant of which is acetate, are likely to contribute to immune regulation, which is important not only within the gut itself, but also systemically and for bone health. Low-dose lactulose has also been shown to enhance the absorption of minerals such as calcium and magnesium from the gut, an effect which may have important implications for bone health. This review provides an overview of the preclinical and clinical evidence published to date showing that low-dose lactulose stimulates the growth of health-promoting gut bacteria, inhibits the growth of pathogenic bacteria, increases the production of beneficial metabolites, improves mineral absorption, and has good overall tolerability. Implications of these data for the use of lactulose as a prebiotic are also discussed.

Keywords: lactulose, prebiotic, mineral absorption, short-chain fatty acid, SCFA, bifidobacteria, gut microbiota, gut health

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INTRODUCTION TO LACTULOSE

History and Clinical Use of Lactulose

Lactulose is an artificial disaccharide composed of galactose and fructose, and is produced via isomerization of lactose (**Figure 1**) (1). Although first described by Montgomery and Hudson in 1929 (2), lactulose gained clinical interest only in 1957, when Petuely discovered that growth of fecal bacteria from the genus *Bifidobacterium* increased following administration of lactulose to infants (3, 4). Because of this activity (i.e., enhancement of bifidobacterial growth), Petuely referred to lactulose as "Der Bifidusfaktor" ("the bifidogenic factor"), a term still in use today (3). Based on the prebiotic and osmotic laxative properties of lactulose, Mayerhofer and Petuely proposed its use to treat constipation in 1959 (5), and lactulose has been used as a laxative for more than 50 years (6).

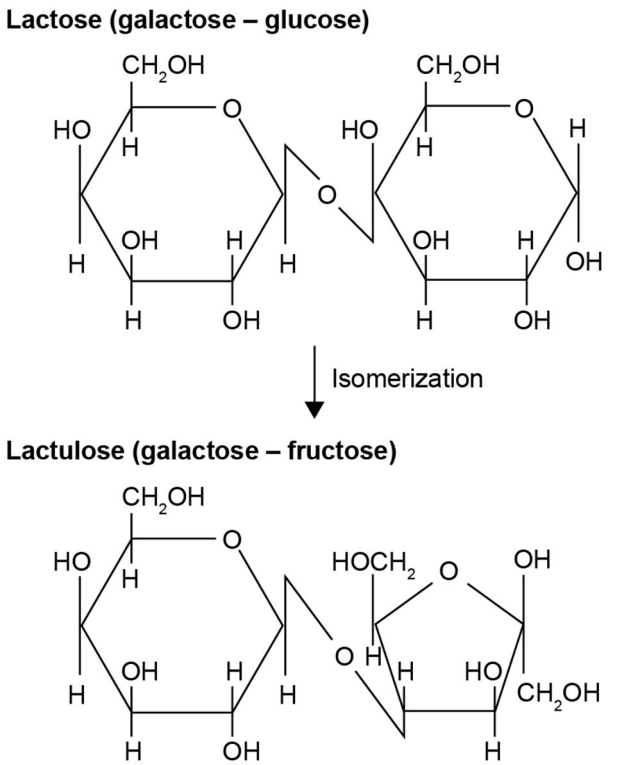

FIGURE 1 | Chemical structure and formation of lactulose (1).

TABLE 1 | Lactulose clinical indications (7).

Indication	Recommended dosing
Constipation in children and adults	• Starting dose of 15–45 mL/day (10–30 g/day) and maintenance dose of 15–30 mL/day (10–20 g/day) in adults and adolescents. • Lower starting and maintenance doses are recommended for: - children aged 7–14 years: 10–15 mL/day (6.7–10.0 g/day) - children aged 1–6 years: 5–10 mL/day (3.3–6.7 g/day) - infants aged < 1 year: up to 5 mL/day (up to 3.3 g/day).
HE in adults	• Starting dose of 30–45 mL (20–30 g) three to four times daily; the maintenance dose may be adjusted to achieve two to three soft stools each day.

HE, hepatic encephalopathy.

In current clinical practice, lactulose is indicated as a laxative for the symptomatic treatment of constipation in children and adults and as a detoxifying agent for the treatment of hepatic encephalopathy (HE) in adults (**Table 1** and **Figure 2**) (7). Although chiefly used for medicinal purposes at medium and high doses for the treatment of constipation and HE, respectively, low-dose lactulose can also be used as a prebiotic to stimulate the growth of health-promoting bacteria in the gastrointestinal (GI) tract, or gut (1, 11). Prebiotics such as lactulose are substrates that are selectively utilized by host microorganisms and that confer a health benefit (12). These can be non-digestible, short-chain carbohydrates that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of colonic bacterial species (13, 14). The numerous beneficial effects of prebiotics are summarized in **Table 2**.

Although the ability of lactulose to stimulate the growth of beneficial gut bacteria has been known for over 60 years (3, 4), lactulose is not commonly recognized as a prebiotic. This review provides an overview of the preclinical and clinical evidence showing that low-dose lactulose confers a health benefit as it:

- stimulates the growth of health-promoting gut bacteria (e.g., *Bifidobacterium* and *Lactobacillus* spp.)
- inhibits the growth of pathogenic bacteria (e.g., certain clostridia)
- increases the production of beneficial metabolites [e.g., short-chain fatty acids (SCFAs)]
- improves mineral absorption
- and has good overall tolerability.

Implications of these data for the use of lactulose as a prebiotic are also discussed.

Mechanism of Action of Lactulose

The treatment effects of lactulose arise from its effects on the gut, namely alteration of colonic microbiota and formation of favorable metabolites (e.g., SCFAs) (**Figure 2**) (4). The human small intestine lacks the enzyme necessary to split the disaccharide lactulose into its component monosaccharides; lactulose, therefore, reaches the large intestine largely intact (1). Once in the colon, lactulose is selectively metabolized by resident colonic microbiota (11), producing SCFAs, intestinal gas (hydrogen, carbon dioxide, and methane) and resulting in increased bacterial mass (1, 11, 34, 35). The ratio of SCFAs produced will be determined by the composition of the host microbiota, as well as the type and quantity of fermentable substrate, pH of the gut, and factors that influence SCFA absorption from the intestine (36).

Acetate, propionate, and butyrate represent the major SCFAs found in the human colon (35). Acetate is the main SCFA produced by fermentation of lactulose (37, 38). Although neither bifidobacteria nor lactobacilli directly produce butyrate upon lactulose fermentation, cross-feeding occurs among the gut microbiota to generate butyrate; members of the genera *Bifidobacterium* and *Lactobacillus* produce acetate and lactate, which are then converted to butyrate by other members of the gut microbiota (39–41).

SCFAs are rapidly absorbed by the colonic epithelium, where they act as substrates for respiration (35), and represent the main carbon flow from the diet through the microbiome to the host (42, 43). Butyrate is the main/preferred source of energy for colonocytes (44, 45). Beyond being fuel for colonocytes, SCFAs have diverse roles in host health, including regulating cells of the immune system, energy storage/metabolism, and gut barrier function (46).

SCFA receptors include G protein-coupled receptors (GPCRs) such as GPR43, GPR41, GPR109A, and OLFR78 (46). SCFAs

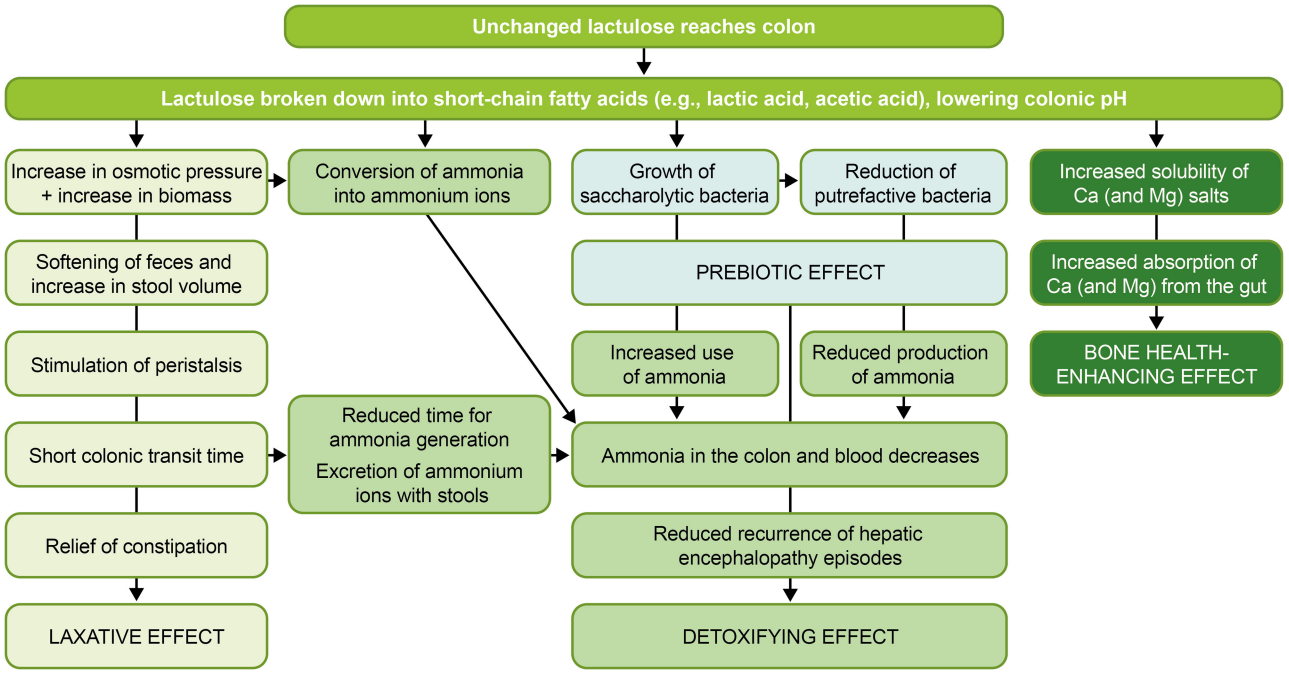

FIGURE 2 | Mechanism of action of lactulose (1, 8–10). Ca, calcium; Mg, magnesium.

and their receptors have several benefits in inflammation; interactions between SCFAs and GPCRs expressed in the gut epithelium and immune cells induce mechanisms that play a key role in maintaining homeostasis in the gut and other organs (47). Acetate has been shown to play an important role in the regulation of inflammation in inflammatory and metabolic diseases and in preventing enteric infection (48, 49). Inflammation is also a major risk factor for cancer development in the digestive tract, and it has been shown that SCFAs, including acetate working through GPR43, act to suppress the development of colorectal cancer (CRC) (46, 50).

SCFAs also have a key role in maintaining a healthy and properly functioning mucosa (37), which is important for nutrient absorption. A properly functioning gut barrier is also vital for preventing the translocation of proinflammatory microbial cell wall components (37). Butyrate has a key role in regulating gut permeability, primarily via orchestration of tight junction proteins (37). Butyrate is also known to induce mucin production, which creates a physical barrier between the colonic microbiota and colonic epithelial cells (51).

Production of acids (e.g., SCFAs) via lactulose fermentation results in a lowering of colonic pH (35). Lowering the gut pH to a level below that at which pathogens are effectively able to compete may help to maintain intestinal homeostasis and to prevent infection (15). An acidic environment also increases the solubility of minerals such as calcium (Ca) and magnesium (Mg) salts, which may represent another means by which lactulose enhances the absorption of these minerals from the gut (8–10).

Use of lactulose as a detoxifying agent for HE mainly stems from the ability of lactulose to alter the gut microbiota to decrease ammonia production and absorption (35). Lactulose also acidifies the colonic content so that ammonia present in the blood diffuses into the colon; here, it is converted into ammonium ions and/or incorporated into microbial biomass and is then excreted via the feces (1, 52). Repression of pathogen colonization with lactulose is also thought to occur from the proliferation of health-promoting gut bacteria and the subsequent competitive effects resulting from their occupation of colonization sites (15).

Growth of resident colonic microbial populations leads to a rise in bacterial biomass (35), and *in-vivo* observations have shown higher fecal bacterial biomass to be associated with shorter intestinal transit times (53). Greater stool volume promotes intestinal peristalsis, accelerating the passage of stool through the colon (1). Increased stool volume may also be achieved via a higher fecal moisture content; metabolism of lactulose increases the osmolality of the intestinal contents (6), exerting an intraluminal osmotic effect and increasing water retention in the lumen (1).

Dose-Dependent Effects of Lactulose

The effects of lactulose are dependent on dose. Depending on the dose used, lactulose can act as a prebiotic, a laxative, or a detoxifying agent (**Figure 3**) (1). Low doses of ∼15 mL/day (∼10 g/day; adult dose) produce a prebiotic effect and enhance Ca and Mg absorption, whereas medium doses of ∼30–60 mL/day (∼20–40 g/day) elicit a laxative effect (used for constipation) and high doses of >90 mL/day (>60 g/day) have a detoxifying effect (used for HE) (1, 4). It is not clear whether these effects are mutually exclusive;

TABLE 2 | Beneficial effects of prebiotics.

Beneficial effect	Description
Altered GM composition	Prebiotics stimulate growth of health-promoting GM species belonging to the genera *Bifidobacterium* and *Lactobacillus* (14)
Enhanced colonization resistance against harmful gut bacteria	Growth of *Bifidobacterium* and *Lactobacillus* populations enhances colonization resistance against pathogens such as *Escherichia coli*, *Clostridium* (e.g., *C. perfringens), Salmonella*, and *Campylobacter* (14–20) Colonization resistance occurs through increased competition for nutrients and normal colonization sites and increased production of endogenous antimicrobial substances that create an inhospitable environment for pathogen growth (15) Inhibiting the growth of these potentially gastroenteritis-causing pathogens thereby increases host resistance to infection (15)
Formation of favorable metabolites	Fermentation of carbohydrates (i.e., prebiotics) by gut bacteria produces SCFAs such as acetate and butyrate, which are associated with several beneficial effects (8, 14) SCFAs are an important respiratory substrate for intestinal epithelial cells, strengthen the gut barrier function, and modulate the immune response (21–23); SCFAs may play a key role in the prevention and treatment of metabolic syndrome, bowel disorders, and CRC (21, 23, 24); SCFAs may help to maintain intestinal homeostasis by lowering the gut pH to a level below that at which pathogens are able to compete effectively (15)
Increased mineral absorption	Prebiotics stimulate absorption of minerals such as Ca, magnesium, zinc, and iron (10, 25) This effect is attributed mainly to luminal acidification by SCFAs, which increases the solubility of minerals, thereby facilitating their absorption through the gut wall; however, several other mechanisms may also be involved (8–10)
Enhanced gut immunity	The GM plays an important role in mediating immune responses at mucosal surfaces; prebiotics can therefore modulate GI immunity (26)
Protection against inflammation-mediated pathologies	Alterations in the normal GM have been implicated in various inflammation-mediated pathologies, including allergic asthma, obesity, type 2 diabetes, Parkinson disease, rheumatoid arthritis, osteoarthritis, and OP (27, 28) Prebiotic-modulated GM may therefore protect against such inflammation-mediated pathologies (27, 28)
Increased mucosal integrity	Prebiotics may help to protect the integrity of the intestinal mucosal barrier; increased crypt depth through stimulation of cellular proliferation, increased villous height, and increased mucin release have been observed after prebiotic intake (9, 18)
Maintenance of bone health	Prebiotics increase Ca absorption (10, 25) The GM has a central role in maintaining bone health and influences bone turnover and density (29) The anti-inflammatory actions of prebiotics may be of particular relevance in the context of bone health, because inflammation disrupts the bone remodeling cycle, leading to bone loss (30) Evidence from animal studies suggests that prebiotics attenuate bone loss through a reduction in systemic inflammation, thereby potentially protecting against age- or menopause-related OP (31–33)

Ca, calcium; CRC, colorectal cancer; GI, gastrointestinal; GM, gut microbiota; OP, osteoporosis; SCFA, short-chain fatty acid.

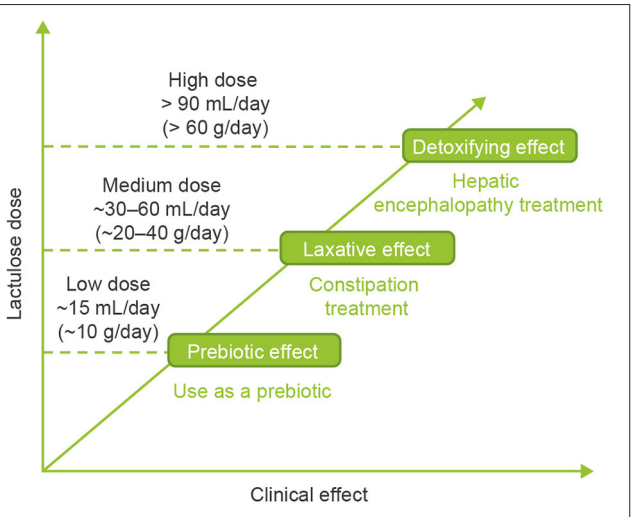

FIGURE 3 | Dose-dependent effects of lactulose (1).

concomitant prebiotic and laxative effects of high-dose lactulose have been demonstrated in patients with chronic idiopathic constipation (54).

LITERATURE SEARCH METHODS

To identify relevant studies of the prebiotic effects of low-dose lactulose, a literature search of the PubMed database was conducted with relevant criteria and a cut-off date of August 31, 2020. Search engines used were EMBASE, Google, MEDLINE, Allied and Complementary Medicine, Analytical Abstracts, BIOSIS Previews, and China/Asia On Demand. A manual search of relevant journals was also performed. A broad search string was used: "lactulose" OR "Duphalac" OR "Bifiteral" OR "Betulac" OR "Lactecon" OR "Avilac" OR "Laktipex" AND "prebiotic" OR "bifidogenic." All publications identified by the search were subsequently reviewed for relevance to the research topic.

EVIDENCE OF THE PREBIOTIC EFFECTS OF LOW-DOSE LACTULOSE

Preclinical Evidence

In vitro, lactulose was a better carbon source than either lactitol or lactose for the major species of intestinal bacteria (55). Lactulose also dose-dependently increased counts of beneficial gut bacteria (including *Bifidobacterium* and *Lactobacillus*) and levels of SCFAs *in vitro* (56). After 120 h, the mean (± standard deviation [SD]) amount of total SCFAs produced with 2, 3, 4, and 5 g/day lactulose was 451 (± 3) mmol, 399 (± 21) mmol,

427 (± 76) mmol, and 471 (± 12) mmol, respectively, compared with 332 (± 34) mmol with control (56). A study in C57BL/6J mice showed that high- and low-dose lactulose increased SCFA production in the intestine, but concentrations differed according to intestinal site and no statistical differences were seen for the main SCFA in feces (57). Interestingly, acetate concentrations were higher in the animals fed with low-dose lactulose at all intestinal sites and in feces, but only statistically significant in the middle colon. Another study in the same mouse model did not show a difference in fecal SCFA when comparing animals fed with high-dose lactulose with control animals, although it did demonstrate a reduction in branched-chain fatty acids in the lactulose-fed group (58). This illustrates the need to carefully consider data from fecal measurements of SCFA, given that concentrations change along the intestinal tract and that SCFA production can be limited by factors other than availability of fermentable substrate. In both studies, lactulose modulated the gut microbiota, increasing the abundance of bifidobacteria and akkermansiae in particular.

Clinical Evidence

Nine clinical trials assessing the prebiotic effects of low-dose lactulose were identified, including a total of 537 participants (16, 54, 59–65). All but two of the studies were conducted in healthy volunteers; 69 of the 304 participants in one study could be considered as having mild constipation (60), and another study was conducted exclusively in individuals with chronic idiopathic constipation ($n = 65$) (54). The trend across the studies was for administration of low-dose lactulose to increase populations of beneficial gut bacteria (e.g., *Bifidobacterium* and *Lactobacillus* spp.) and metabolites (e.g., SCFAs), to reduce harmful gut bacteria (e.g., certain clostridia) and to lower fecal pH (**Table 3**).

In an open-label, single-arm study, eight healthy volunteers received a once-daily drink containing 3 g of lactulose for 2 weeks, in addition to their normal diet (63). During the lactulose intake period, the number of bifidobacteria increased significantly compared with values before intake; mean (± SD) $\log_{10}$ cells/g feces was 9.7 (± 0.1) before treatment (day 0) compared with 10.4 (± 0.1) $\log_{10}$ cells/g feces on day 7 of intake. Conversely, the numbers of lecithinase-positive clostridia, including *Clostridium perfringens*, and Bacteroidaceae decreased slightly but significantly compared with values before intake (63). After 7–14 days of treatment, lactulose also significantly reduced the levels of potentially toxic substances, including fecal indole and phenol, and significantly reduced activities of fecal β-glucuronidase, nitroreductase, and azoreductase. Finally, lactulose contributed to improvements in the intestinal environment; by day 14 of intake, mean fecal pH decreased from 7.0 to 6.4 and mean water content increased by 3.5–5.3% (63).

In a placebo (PBO)-controlled randomized clinical trial (RCT), 20 healthy volunteers received either lactulose 10 g/day or glucose/lactose (PBO) for between 26 and 33 days (64). Lactulose significantly increased populations of *Bifidobacterium* spp. compared with pre-treatment levels; mean (± SD) $\log_{10}$ cells/g feces was 8.8 (± 0.5) before treatment compared with 9.3 (± 0.3) $\log_{10}$ cells/g feces after treatment (**Figure 4A**). This increase was also significant compared with the changes in *Bifidobacterium* spp. population levels that occurred with PBO over the same period. The effect was most pronounced in individuals with the lowest pre-treatment *Bifidobacterium* spp. population counts. There was a significant reduction in levels of *Clostridium* spp. during lactulose intake, from 8.1 (± 0.5) $\log_{10}$ cells/g feces before treatment to 7.7 (± 0.4) $\log_{10}$ cells/g feces after treatment (**Figure 4B**). No significant differences in population levels of *Clostridium* spp. were observed in the PBO group over the treatment period or between the lactulose and PBO groups (64).

A parallel-group, PBO-controlled RCT was carried out to assess the effects of prolonged low-dose lactulose on fecal bifidobacteria (59). Sixteen healthy volunteers were randomized to lactulose 10 g/day or sucrose (PBO) for 6 weeks. Fecal bifidobacterial counts were significantly higher after prolonged low-dose lactulose ingestion than after PBO ingestion. Lactulose led to significantly increased fecal *Bifidobacterium* counts from days 0 to 21 and day 42 [mean ± standard error of the mean, 8.25 ± 0.53, 8.96 ± 0.40, and 9.54 ± 0.28 log colony-forming units (CFU)/g wet weight, respectively] (59). Throughout the study, total anaerobes, *Lactobacillus* spp., pH, and other variables did not change significantly in either group (59).

In another RCT, 36 healthy volunteers were randomized to either lactulose 20 g/day, lactitol 20 g/day, or sucrose/lactose (PBO) for 4 weeks (16). Lactulose and lactitol significantly increased populations of *Bifidobacterium*, *Lactobacillus*, and *Streptococcus* spp. by 3.0, 1.9, and 1.2 log CFU, and 1.4, 0.7, and 0.6 log CFU, respectively. Lactulose and lactitol significantly decreased populations of *Bacteroides* spp., *Clostridium* spp., coliforms, and *Eubacterium* spp. by 4.1, 2.3, 1.8, and 3.0 log CFU, and 1.5, 1.2, 1.0, and 1.9 log CFU, respectively (16). Beneficial changes were greater with lactulose than with lactitol, and the onset of effect was more rapid with lactulose (1 vs. 2 weeks with lactitol) (16). Lactulose and lactitol both led to significant changes in fecal biochemistry (pH, fecal moisture, and SCFAs) compared with PBO (16).

An open-label, single-arm, "before-after" study in 26 healthy Japanese women consisted of a pre-observation period followed by three 2-week ingestion periods with a 2-week washout period between each (61). Across the three ingestion periods, volunteers received escalating doses of lactulose (1, 2, and 3 g/day). Compared with the pre-observation/washout periods, fecal bifidobacterial counts, defecation frequency, and number of defecation days significantly and dose-dependently increased following intake of lactulose 1, 2, and 3 g/day (61). The mean (± SD) number of bifidobacteria significantly increased from 9.93 (± 0.57) log CFU/g feces to 10.10 (± 0.40) log CFU/g feces with lactulose 1 g/day, from 9.95 (± 0.63) log CFU/g feces to 10.23 (± 0.53) log CFU/g feces with lactulose 2 g/day, and from 10.09 (± 0.51) log CFU/g feces to 10.38 (± 0.28) log CFU/g feces with lactulose 3 g/day. These results suggest that doses of lactulose as low as 1 g/day can exert a prebiotic effect (61).

The same study team conducted a crossover RCT in 52 healthy Japanese women (62). Volunteers were randomized to lactulose 2 g/day or glucose (PBO) for 2 weeks. After a 3-week washout period, participants were crossed over to the

TABLE 3 | Summary of key efficacy findings from clinical studies of the prebiotic and mineral absorption effects of low-dose lactulose.

Study population, age	*N*	Aims	Design	Treatment regimen	Key efficacy findings	References
Prebiotic effects						
Healthy M and F 8–22 y	8	Assess the effects of lactulose on the composition and metabolic activity of fecal microbiota	Open-label, single-arm study	Lactulose 3 g/D for 2 W	↑ (~7%) in populations of bifidobacteria ($p < 0.001$) ↓ (slight) in populations of Bacteroidaceae and lecithinase-positive clostridia (both $p < 0.05$) ↓ fecal indole, phenol (both $p < 0.05$), and skatol (in 4/8 volunteers) ↓ β-glucuronidase, nitroreductase, and azoreductase activities (all $p < 0.05$) ↓ fecal pH 7.0 → 6.4 ↑ fecal moisture content by 4.3–5.3%	(63)
Healthy[a] M and F 13–66 y	304	Assess the effects of lactulose on intestinal function and fecal character	Open-label study in three groups	$n = 8$ healthy volunteers: lactulose 4 g/D for 3 W $n = 296$[a]: lactulose 3 or 5 g/D for 10 D	Lactulose 4 g/D: ↑ populations of bifidobacteria ($p < 0.05$) ↓ populations of Bacteroidaceae, eubacteria, and clostridia (mean ratio to total bacteria when compared with after lactulose intake; all $p < 0.05$) ↓ fecal pH (compared with after lactulose intake; $p < 0.05$) ↓ fecal indole (compared with before lactulose intake; $p < 0.05$) Lactulose 3 or 5 g/D: ↑ defecation frequency and feces became more watery, yellowish, and softer (compared with before and after lactulose intake for both 3 and 5 g/D groups; $p < 0.05$ or $p < 0.01$, respectively) Results were consistent between patients with low and normal defecation frequencies	(60)
Healthy[b] 18–50 y	20	Assess the effects of lactulose on colonic microbiota	Randomized, double-blind, PBO-controlled study	R 1:1 to lactulose 10 g/D ($n = 10$) or PBO (glucose/lactose; $n = 10$) for 26–33 D	With lactulose: ↑ populations of *Bifidobacterium* spp. vs. pre-tx levels ($p < 0.01$) ↑ populations of *Bifidobacterium* spp. vs. PBO ($p < 0.01$) ↓ populations of *Clostridium* spp. vs. pre-tx levels ($p < 0.01$)	(64)
Healthy M and F 19–42 y	16	Assess the effects of prolonged lactulose on fecal bifidobacteria and metabolic indices potentially involved in colonic carcinogenesis	Randomized, double-blind, PBO-controlled, parallel-group study	R 1:1 to lactulose 10 g/D ($n = 8$) or PBO (sucrose; $n = 8$) for 6 W	With lactulose: ↑ fecal *Bifidobacterium* counts from D0 to D21 and D42 ($p = 0.048$) and vs. PBO ($p = 0.03$) Neither group showed significant changes in total anaerobes, lactobacilli, pH, or other study variables	(59)
Healthy[b] 24–31 y	36	Assess the comparative efficacy of lactulose and lactitol on colonic microbiota and fecal biochemistry	Randomized, double-blind, PBO-controlled study	R 1:1:1 to lactulose 20 g/D ($n = 12$), lactitol 20 g/D ($n = 12$), or PBO (sucrose/lactose; $n = 12$) for 4 W	Lactulose vs. PBO: ↑ populations of probiotic bacteria ($p < 0.01$) ↓ populations of putrefactive bacteria ($p < 0.01$) Beneficial changes greater with lactulose vs. lactitol Effect onset more rapid with lactulose vs. lactitol (1 vs. 2 W) Both lactulose and lactitol led to significant changes in fecal biochemistry compared with PBO	(16)

(Continued)

TABLE 3 | Continued

Study population, age	*N*	Aims	Design	Treatment regimen	Key efficacy findings	References
Healthy postmenopausal F 55–64 y	10	Assess the effects of lactulose on intestinal microbiota and SCFA production	*In-vivo* effect on fecal samples and computer-controlled *in-vitro* model of the proximal large intestine	Lactulose 10 g/D for 7 D. Microbiota obtained from volunteers before and after lactulose consumption were adapted to an *in-vitro* model of the proximal colon and then fed lactulose 10 g/D introduced gradually over a 48-h period	Following *in-vivo* lactulose consumption: no changes in fecal pH, dry weight, or mean molar SCFA ratios in the fecal samples ↑ populations of *Bifidobacterium* ($p < 0.05$) Following adaptation of the *in-vivo* samples (before and after lactulose consumption) to the *in-vitro* culture system: clear effect of *in-vivo* lactulose consumption on *Lactobacillus* and *Enterococcus* (both ↑) clear effect of *in-vivo* lactulose consumption on SCFA ratios (↓ butyrate; $p < 0.001$)	(65)
Healthy F 18–21 y	26	Assess the prebiotic effects of lactulose on defecation frequency	Open-label, single-arm, before-after study	Lactulose 1, 2, and 3 g/D for 2 W each. Crossed over after a 2-W washout period	Lactulose 1, 2, and 3 g/D: ↑ defecation frequency ↑ defecation D ↑ fecal bifidobacteria counts	(61)
Healthy F mean (± SD): 20.2 (± 2.4) y	52	Assess the prebiotic effects of lactulose on defecation frequency	Randomized, double-blind, PBO-controlled, crossover study	R 1:1 to lactulose 2 g/D or PBO (glucose) for 2 W. Crossed over after a 3-W washout period	Lactulose vs. PBO: ↑ populations of *Bifidobacterium* in feces ↑ proportion of *Bifidobacterium* in feces ↑ defecation frequency ↑ number of defecation D improved fecal character (consistency and volume)	(62)
Chronically constipated M and F mean (± SD): 57 (± 18) y	65	Assess the comparative efficacy of lactulose and PEG-4000 on colonic microbiota	Prospective, multicenter, randomized, single-blind, active-controlled, parallel-group study	R 1:1 to lactulose or PEG-4,000 for 4 W. W1 dose fixed (20 g/D); W2 dose could vary (10–30 g/D); W3–4 dose fixed (10–30 g/D)	With lactulose (D −1 to D28): ↑ populations of fecal bifidobacteria and anaerobes ($p < 0.02$) no significant differences in SCFAs With PEG-4000 (D−1 to D28): no significant differences in populations of fecal bifidobacteria/anaerobes ↓ total SCFAs ($p = 0.02$), acetate ($p = 0.02$), and butyrate ($p = 0.04$)	(54)
Mineral absorption effects						
Healthy M 23–42 y	24	Assess the effect of lactulose on Ca and Mg absorption	Randomized, double-blind, three-period, three-group crossover study	R 1:1:1 to PBO, lactulose 2 or 4 g/D, plus $CaCO_3$ 300 mg (20 mg ^{44}Ca) and MgO 150 mg (28 mg ^{25}Mg). Crossed over after a 2-W washout period between each tx	Lactulose 2 or 4 g/D enhanced Ca and Mg absorption vs. PBO Urinary stable isotopes ratios ($^{44}Ca/^{40}Ca$ and $^{25}Mg/^{24}Mg$) ↑ with lactulose dose and were significantly different for lactulose vs. PBO for Ca (lactulose 4 g/D) and Mg (lactulose 2 and 4 g/D) (all $p < 0.01$)	(66)
Healthy postmenopausal F 56–64 y	12	Assess the effect of lactulose on Ca absorption	Randomized, double-blind, PBO-controlled crossover study	R 1:1:1 to lactulose 5 or 10 g/D or PBO (aspartame) for 9 D. ^{44}Ca and ^{48}Ca given on D8. Crossed over after a 19-D washout period between each tx	Lactulose 5 or 10 g/D dose-dependently ↑ intestinal Ca absorption without ↑ urinary excretion	(67)
Postmenopausal, with osteopenia F 52–67 y	41	Assess the effect of lactulose on BMD maintenance	Randomized, double-blind, PBO-controlled parallel-group study	R 1:1 to lactulose 10 g/D plus $CaCO_3$ 500 mg/D or PBO plus $CaCO_3$ 1,000 mg/D for 12 months	Lactulose plus $CaCO_3$ 500 mg/D was as effective as lactulose plus $CaCO_3$ 1,000 mg/D	(68)

[a] *69 patients could be considered mildly constipated (defecation frequency < 1.0/D).*

[b] *Sex of participants not stated.*

↓, decreased; ↑, increased; BMD, bone mineral density; Ca, calcium; D, day(s); F, female; M, male; Mg, magnesium; n, number of participants; N, total number of participants; PBO, placebo; PEG-4000, polyethylene glycol-4000; R, randomized; SCFA, short-chain fatty acid; SD, standard deviation; spp., species (plural); tx, treatment; W, week(s); y, years.

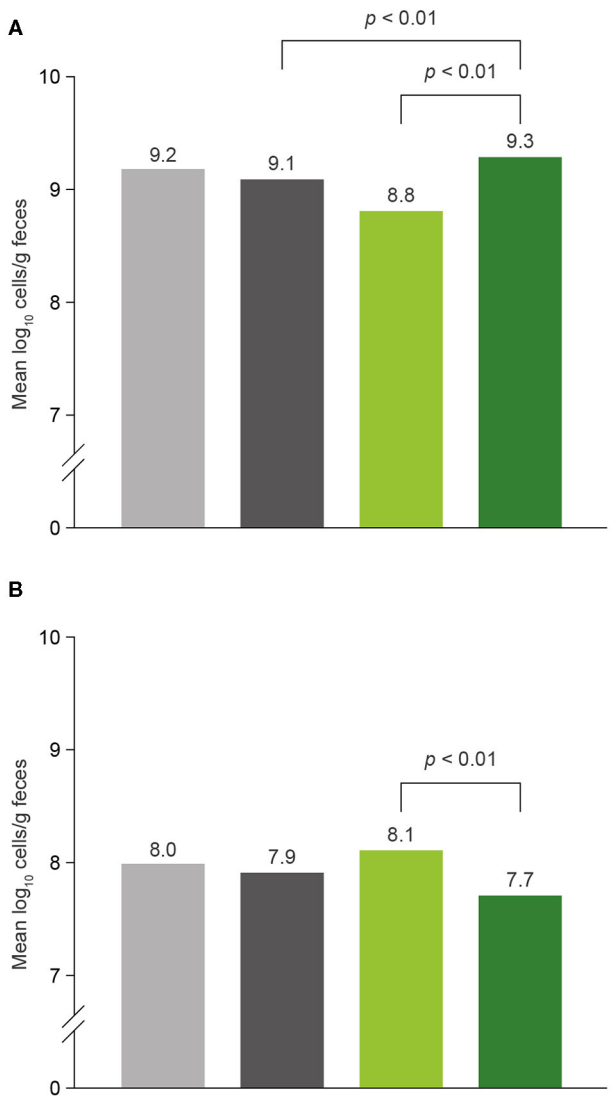

FIGURE 4 | Impact of lactulose 10 g/day and placebo on **(A)** *Bifidobacterium* and **(B)** *Clostridium* counts (assessed by FISH) (64). $n = 20$. FISH, fluorescence *in-situ* hybridization.

other treatment group. The mean (± standard error) number of bifidobacteria in feces was significantly higher with lactulose compared with PBO [9.53 (± 0.06) vs. 9.16 (± 0.06) log CFU/g feces, respectively] (62). The proportion of *Bifidobacterium* spp. in feces was also significantly higher after lactulose than after PBO treatment [25.3% (± 1.4%) vs. 18.2% (± 1.4%)]. Moreover, lactulose administration also increased defecation frequency and the number of defecation days, and improved fecal consistency compared with PBO (62).

The only study conducted in postmenopausal women compared the effect of lactulose on fecal parameters *in vivo* with the effect in an *in-vitro* model of the proximal large intestine (65). Fecal samples from 10 healthy postmenopausal volunteers were collected before and after 7 days receiving lactulose 10 g/day. In the *in-vitro* model, lactulose 10 g/day was fed to microbiota over a 48-h period (65). Lactulose promoted *Bifidobacterium* growth *in vivo* and *Lactobacillus* and *Enterococcus* spp. growth *in vitro* (65). No changes in fecal pH, dry weight, or mean molar SCFA ratios were observed in the *in-vivo* fecal samples. However, there was a clear effect on SCFA ratios in the *in-vitro* model, with lactulose causing a pronounced reduction of butyrate by the postmenopausal microbiota (65). The authors concluded that the *in-vitro* model provided a better reflection of the effects of lactulose fermentation in the proximal colon in terms of microbial composition changes and metabolite production, and that, *in vivo*, feces do not closely reflect proximal colon fermentation but a summation of microbiota-related activities from proximal to distal colon (65).

An open-label study consisted of 304 Japanese volunteers split across three lactulose dose groups (60). In the first group, eight healthy volunteers received lactulose 4 g/day for 3 weeks. The remaining 296 participants were divided into two groups, distributed evenly with respect to age and sex, and received either lactulose 3 or 5 g/day for 10 days (60). Of the 296 participants who received lactulose 3 or 5 g/day, 69 had low stool frequency and could therefore be considered as having mild constipation (60). At a dose of 4 g/day, lactulose significantly increased bifidobacterial populations; the ratio of bifidobacteria to total bacteria increased from 22.4% before lactulose intake to 50.5% during intake. Corresponding Bacteroidaceae, eubacteria, and clostridia populations decreased significantly; the proportion of Bacteroidaceae, for example, decreased from 48.4% before lactulose treatment to 28.8% after treatment (60). At 4 g/day, lactulose significantly increased defecation frequency (0.83/day before intake vs. 0.95/day during intake), reduced fecal pH (6.33 during intake vs. 6.52 after intake; no significant difference during intake vs. before intake) and reduced fecal indole (70.3 μmol/g feces before intake vs. 38.7 μmol/g feces during intake). At 3 or 5 g/day, lactulose resulted in a significant increase in defecation frequency and the feces became more watery, yellowish, and softer. Results were consistent between individuals with low defecation frequency and those with normal defecation frequency (60).

Finally, a single-blind RCT compared the effect of lactulose with that of another osmotic laxative, polyethylene glycol 4000 (PEG-4000) on colonic microbiota. This was the only active-comparator RCT identified and the only study conducted in individuals with chronic constipation ($n = 65$) (54). The diagnosis of chronic idiopathic constipation was based on the Rome I diagnostic criteria of constipation: the presence for at least 6 months of fewer than three stools per week and/or difficulty in defecation and/or straining on passage of stool (54). Lactulose or PEG-4000 was given at a dosage of 20 g/day for the first week. During week 2, dose adjustments were permitted depending on the efficacy and tolerance of lactulose (allowing a dose of 10–30 g/day) (54). Following dose adjustment, the investigator fixed the dose for the last 2

weeks. From days −1 to 28, median fecal bifidobacteria and anaerobe counts increased significantly with lactulose (from 8.4 to 9.1 log CFU/g wet weight and from 10.6 to 10.9 log CFU/g wet weight, respectively), whereas no significant changes were observed with PEG-4000 (54). Over the same time period, metabolic activity of fecal microbiota was strongly inhibited with PEG-4000; there was a significant decrease in levels of total SCFAs, butyrate, and acetate. No significant differences in levels of SCFAs were noted with lactulose (54), and no differences were seen in either treatment group in fecal pH or in fecal counts of *Lactobacillus*, clostridial spores, *Bacteroides*, or enterobacteria (54).

Taken together, the results of clinical studies published to date, consistent with preclinical data, show that low-dose lactulose increases counts of *Bifidobacterium* and *Lactobacillus* spp. and beneficial SCFAs, reduces the growth of harmful gut bacteria (e.g., certain clostridia), and lowers fecal pH.

EVIDENCE OF THE MINERAL ABSORPTION EFFECTS OF LOW-DOSE LACTULOSE

Preclinical Evidence

Preclinical studies have shown that lactulose stimulates Ca and Mg absorption from the gut in rats, an effect that appears to occur in both the small intestine and the cecum (25, 69–71).

Clinical Evidence

In separate clinical studies, low-dose lactulose has been shown to enhance mineral absorption in healthy men (66) and in postmenopausal women (**Table 3**) (67).

A randomized, double-blind, three-group crossover study was conducted in 24 healthy men (23–42 years old) to evaluate the effect of lactulose on Ca and Mg absorption (66). Volunteers received test food containing lactulose 0 g (PBO), 2 or 4 g together with $CaCO_3$ 300 mg (containing 20 mg of ^{44}Ca) and MgO 150 mg (containing 28 mg of ^{25}Mg). Participants crossed over to each of the other two lactulose doses, with a 2-week washout period between each treatment. Results showed that the higher the dose of lactulose, the higher the urinary stable isotopes ratios ($^{44}Ca/^{40}Ca$ and $^{25}Mg/^{24}Mg$). This difference was significant for Ca between PBO and lactulose 4 g and for the Mg ratio between PBO and both doses of lactulose. This study demonstrates that low-dose lactulose enhances the absorption of Ca and Mg in healthy men and that it does so in a dose-dependent manner (66).

A similar dose-dependent increase in Ca absorption with lactulose was observed in a randomized, double-blind, PBO-controlled crossover study in 12 healthy postmenopausal women (aged 56–64 years) (67). Participants drank 100 mL of water containing lactulose 5 or 10 g or PBO for 9 days. Oral ^{44}Ca and intravenous ^{48}Ca were administered on day 8 of treatment, and urine isotope measurements were used to calculate Ca absorption. A 19-day washout period separated each treatment. Mean (± SD) Ca absorption with PBO, lactulose 5 g/day, and lactulose 10 g/day was 27.7% (± 7.7%), 30.0% (± 7.6%), and 32.2% (± 7.0%), respectively. The difference in Ca absorption between lactulose 10 g/day and PBO was significant (**Figure 5**) (67).

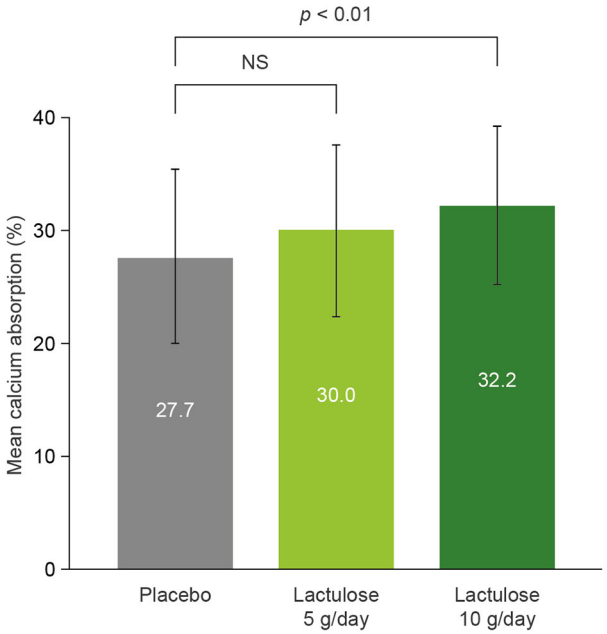

FIGURE 5 | The effect of lactulose on calcium absorption in healthy postmenopausal women (67). $n = 12$. Error bars show ± standard deviation. NS, not significant.

The chronic effect of lactulose use on maintenance of bone mineral density (BMD) has also been assessed in postmenopausal women with osteopenia (68). In a randomized, double-blind, PBO-controlled parallel-group study, 41 women received either lactulose 10 g, vitamin D3 400 IU, and $CaCO_3$ 500 mg, or PBO, vitamin D3 400 IU plus $CaCO_3$ 1,000 mg once daily for 12 months. Baseline daily Ca intake was similar in both treatment arms. Differences in least-square means of BMD (measured in the lumbar spine) between lactulose and PBO at final visit were not statistically significant. The results suggest that lactulose may help to maintain BMD in postmenopausal women by increasing Ca absorption (68).

EVIDENCE OF THE SAFETY PROFILE OF LOW-DOSE LACTULOSE

Lactulose is absorbed in insignificant amounts in the gut, which then undergo rapid excretion by the kidneys; the direct effects of lactulose, therefore, remain localized to the gut (72). Of the 11 clinical studies of the prebiotic/mineral-absorption effects of low-dose lactulose included in this review, eight reported safety outcomes [from a total of 519 participants (385 healthy volunteers; 69 with mild constipation, with defecation frequency < 1.0/day; 65 with chronic constipation)] (54, 59–62, 64, 66, 67). These studies demonstrated that low-dose lactulose (1–10 g/day)

TABLE 4 | Summary of key safety/tolerability findings from clinical studies of lactulose.

Design and study population	Key safety findings	References
Prebiotic effects of lactulose		
Open-label study in 304 healthy adult volunteers (one group non-constipated; one group mildly constipated)	Lactulose was generally well-tolerated at all doses Most participants reported that treatment had no significant tolerability effect (59–80% of all abdominal symptom comments were "nothing significant") However, small increases in abdominal gaseous symptoms (flatulence, abdominal distension, passing flatus) were observed in both treatment groups	(60)
Randomized, double-blind, PBO-controlled study in 20 healthy adult volunteers	Lactulose 10 g/day was generally well-tolerated One participant reported a moderate to severe change in flatulence, bloating, and accompanying abdominal pain	(64)
Randomized, double-blind, PBO-controlled, parallel-group study in 16 healthy adult volunteers	Prolonged low-dose lactulose (10 g/day) was well-tolerated and was associated with mild digestive symptoms Excess flatus was more common in the lactulose group vs. PBO ($p = 0.03$) but was very mild. Bloating, borborygmi, and abdominal pain did not differ between the groups	(59)
Open-label, single-arm, before-after study in 26 healthy women	Low-dose lactulose (1, 2, and 3 g/day) was well-tolerated No side effects or SAEs were reported Secondary abdominal symptoms were predominantly GI in nature; however, their incidence did not differ significantly between pre-observation/washout periods and respective lactulose intake periods	(61)
Randomized, double-blind, PBO-controlled, crossover study in 52 healthy women	No side effects or SAEs were reported The main tolerability symptoms were GI in nature, but these were similar for low-dose lactulose (2 g/day) and PBO	(62)
Prospective, multicenter, randomized, single-blind, active-controlled, parallel-group study in 65 adults with chronic constipation	The proportion of patients reporting at least 1 day of moderate-to-severe borborygmi and bloating decreased in both the lactulose and PEG-4000 treatment groups Eight patients in the lactulose group and five in the PEG-4000 group reported a total of 17 AEs (events assessed included borborygmi, bloating, abdominal pain, and excess flatus); however, there were no SAEs	(54)
Mineral absorption effects of lactulose		
Randomized, double-blind, three-period, three-group crossover study in 24 healthy men	Low-dose lactulose (2 or 4 g/day) was well-tolerated, with no side effects reported	(66)
Randomized, double-blind, PBO-controlled crossover study in 12 healthy postmenopausal women	Low-dose lactulose (5 and 10 g/day) was well-tolerated There were no significant differences in GI complaints between low-dose lactulose and aspartame PBO treatment in a postmenopausal population	(67)

AE, adverse event; GI, gastrointestinal; PBO, placebo; PEG-4000, polyethylene glycol-4000; SAE, serious adverse event.

is well-tolerated in healthy adults (including postmenopausal women) and adults with constipation, with few mild to severe abdominal/GI symptoms or other adverse effects (**Table 4**). GI symptoms seen with lactulose are dose-dependent; the higher the dose, the greater the incidence of symptoms such as abdominal pain, bloating, and diarrhea (73). At the dose relevant for its use as a prebiotic, as per the eight studies that reported safety outcomes, lactulose is generally associated with mild digestive symptoms, such as small increases in flatulence and abdominal distension/bloating (54, 59–62, 64, 66, 67). Furthermore, when GI symptoms do occur, they usually remit spontaneously within a few days of starting treatment or upon dose reduction (72).

DISCUSSION

The studies included in this review clearly demonstrate that the prebiotic health benefits of lactulose extend beyond a simple osmotic laxative effect observed at higher doses; evidence shows that low-dose lactulose stimulates the proliferation of *Bifidobacterium* and *Lactobacillus* spp. and the production of SCFAs, reduces levels of harmful gut bacteria (e.g., certain clostridia), and improves mineral absorption. Low-dose lactulose is also well-tolerated, with few mild to severe abdominal/GI symptoms or other adverse effects.

Extrapolation of the Results to Special Populations

It is important to note that the studies included in this review were conducted mostly in healthy adult volunteers; outcomes in special populations and non-healthy individuals may therefore differ from those reported here. Two studies were conducted exclusively in postmenopausal women (65, 67), and two other studies included patients with constipation (54, 60); however, none included special populations such as women who were pregnant or lactating, children, or the elderly. Nevertheless, when used at higher doses than investigated here (i.e., for chronic constipation or HE), lactulose has demonstrated a favorable safety profile in these populations (74). Similarly, although patients with diabetes were not included in these studies, it has been shown that blood glucose levels remain unchanged after lactulose intake in healthy volunteers, suggesting that lactulose as a functional food ingredient may also be consumed by people with impaired glucose tolerance (75). The effects of lactulose established in healthy individuals cannot, however,

be extrapolated reliably to patients with certain diseases, such as irritable bowel syndrome, liver disease (e.g., cirrhosis), and chronic kidney disease (76). Although low-to-medium doses of lactulose (10–30 g/day) were shown to have prebiotic effects in patients with chronic idiopathic constipation (54), there is a notable lack of data on the prebiotic effects of lactulose in GI disorders other than constipation. There is therefore a need for separate studies of the effect of lactulose on the composition of the gut microbiota in patients with different pathologies (76).

Lactulose Dose Considerations

Given the dose-dependent nature of GI symptoms, the higher the dose of lactulose, the more likely patients are to experience diarrhea (72). Concerning the addition of lactulose to infant formula milk, the incorporation of 0.5% lactulose is considered adequate to stimulate bifidobacterial growth to the extent observed in breast-fed babies, while preventing any laxative action due to lactulose (77). The transitory laxative threshold for lactulose has been estimated to be 0.26 g/kg body weight, which indicates that it would be acceptable to administer lactulose at a dose of up to 13 g/day for a person weighing 50 kg; doses beyond this threshold are more likely to induce diarrhea (78). The European Food Safety Authority recognizes lactulose at a dose of 10 g/day as a food supplement and supports the claim that daily consumption of lactulose at this dose brings about "a reduction in intestinal transit time" (79).

The dose range of 1–10 g/day of lactulose used in the clinical studies included in this review is too broad a range to be practical. We therefore suggest that, for use as a prebiotic in adults, a dose of 5–10 g/day of lactulose is likely to provide a positive benefit–risk ratio while being practical and convenient for the patient.

Potential Benefits of Low-Dose Lactulose

Mineral Absorption and Bone Health

Two studies in this review, including one in healthy postmenopausal women, demonstrated that lower doses of lactulose increase the absorption of minerals from the gut (66, 67). The increased absorption of Ca and Mg with lactulose treatment appears to occur primarily in the small intestine, with some evidence that it may also take place in the cecum (25). Increased absorption of Ca, in particular, may have important implications for maintaining or improving bone density. The bone-health-supporting potential of prebiotics such as lactulose will depend on the host's characteristics, such as their age, postmenopausal status, and capacity to absorb Ca (9). Individuals who have a high demand for Ca (e.g., those who are going through puberty or are postmenopausal) are more likely to benefit from prebiotics than healthy adults (9). During bone development, which typically takes place during adolescence but can continue into early adulthood, BMD increases until peak bone mass is reached (80). Importantly, peak bone mass is a key determinant of osteoporosis later in life (81). Given the critical role of Ca in bone formation and the importance of the increase in BMD that occurs during bone development, lactulose may have a role in ensuring adequate Ca intake during this crucial period.

Because Ca absorption declines with age, older patients could also derive particular benefit from low-dose lactulose treatment (82, 83). In particular, women experience a rapid decline in intestinal Ca absorption with the onset of menopause (82, 84). Declining estrogen levels that occur with menopause lead to increased bone turnover, with resorption exceeding formation (31, 85, 86), resulting in rapid bone loss and risk of menopausal osteoporosis (31). Because bone loss in recently postmenopausal women is largely influenced by a decline in circulating estrogen, women who are beyond menopause by more than 6 years may benefit more from lactulose than women who are recently postmenopausal (9). The potential bone-health-enhancing effects of lactulose and the populations likely to benefit most from increased Ca absorption require further investigation.

Similarly, there is a growing realization that inflammation has a significant influence on bone turnover and increases the risk of osteoporosis and other bone and joint chronic pathologies (67, 87). The potential of SCFAs, especially acetate and butyrate, to regulate inflammatory processes both in the gut and systemically therefore raises the intriguing possibility of managing bone health through prebiotics such as lactulose. Studies in mice have shown that treatment with SCFAs and feeding with a high-fiber diet significantly increase bone mass and prevent postmenopausal and inflammation-induced bone loss (88). SCFAs were identified as potent regulators of osteoclast metabolism and bone homeostasis (88).

Potential Role of Lactulose as a Prebiotic

At present, lactulose is available as a medicinal product (at medium and high doses for the treatment of constipation and HE, respectively) and at a low dose as a food supplement. Despite lactulose not being widely recognized as a prebiotic, its prebiotic effects are outlined in the pharmacodynamic section of its prescribing information (7). Asian constipation treatment guidelines also highlight the prebiotic effects of lactulose and state that the ability of lactulose to stimulate the growth of health-promoting bacteria in the human gut could contribute to an improvement in bowel function (74, 89).

As reported in this review, data published to date demonstrate that lactulose at a dose of 5–10 g/day exerts prebiotic effects, contributing to a healthy gut environment and increasing mineral absorption. This appears to support both the preventive and the therapeutic use of low-dose lactulose as a prebiotic to improve gut health and to ensure a guaranteed uptake of Ca. Through its potential bone-health-enhancing effects, low-dose lactulose may have a role in combating age- or menopause-associated osteoporosis. Furthermore, given the potential immune-enhancing effects of prebiotics, low-dose lactulose might also prove a useful dietary additive for individuals genetically predisposed to CRC, as well as for the prevention and treatment of other inflammation-mediated pathologies. Further studies are required to test this hypothesis.

Immune Modulatory Potential of SCFAs

GPR43, which is a SCFA GPCR, plays a key role in intestinal inflammatory responses in health and disease (90),

and the interactions of SCFAs with GPR43 are pivotal in suppressing/resolving colonic inflammation (91). GPR43 is the pre-eminent receptor for acetate in the intestinal setting, although acetate has been shown to activate other GPCRs, such as GPR41 (90). Acetate, the main SCFA generated from lactulose fermentation, acts via GPR43 to regulate immune function, to enhance the inflammatory response against pathogens in the gut, and to regulate/resolve inflammation elsewhere in the body (48, 49). The systemic action of acetate may have important implications for immune-mediated diseases (e.g., cancer, metabolic syndrome, dementia, and autoimmune diseases) and for bone health.

Colorectal Cancer Protection

The modulation of gut microbiota represents a novel strategy for the prevention of CRC and the optimization of its treatment (92). A causal relationship exists between intestinal microbial dysbiosis and CRC pathogenesis, whereby several bacterial species have been identified as contributing to colorectal proliferation (e.g., *Fusobacterium nucleatum*, *Peptostreptococcus anaerobius*, and enterotoxigenic *Bacteroides fragilis*) whereas others (e.g., *Lachnospiraceae* spp., *Bifidobacterium animalis*, and *Streptococcus thermophilus*) have been found to be depleted in patients with CRC (92). This suggests that these depleted bacteria may exert a protective effect against CRC. The use of prebiotics to stimulate the colonic abundance and activity of these health-promoting bacteria or to achieve a direct anti-inflammatory effect on the gut represents a promising therapeutic strategy (92).

Butyrate has been shown to modulate the expression of genes involved in the defense against oxidative and metabolic stress in primary human colon cells *in vitro* (21, 24). This suggests that butyrate-induced changes in gene expression could protect colon cells from oxidative stress and suppress inflammatory reactions known to increase the risk of CRC (24). An *in-vitro* study in colonic macrophages and dendritic cells demonstrated that signaling via the GPR109A receptor, a receptor for butyrate in the colon, promoted anti-inflammatory properties (93). Further, GPR109A deficiency in mice was shown to promote colon carcinogenesis whereas GPR109A activation suppressed colonic inflammation and carcinogenesis (93). Acetate may also have protective effects against CRC, acting via its receptor GPR43 to regulate the inflammation involved in intestinal carcinogenesis (50, 90).

Thus, through promoting the growth of *Bifidobacterium* and the subsequent positive impact on levels of acetate and butyrate, lactulose could feasibly protect against the development of CRC. It should be noted that the suggested inhibitory effect of SCFAs on cancer is not completely understood and further studies are needed into the effects of lactulose on CRC (65).

Limitations of the Review

Although the literature search to identify studies of interest was in-depth, a systematic approach was not adopted, and it is therefore possible that not all studies on the prebiotic properties of lactulose have been considered. In addition, studies in the field of prebiotics employ a wide variety of microbiological methodologies, model systems, and bacterial nomenclature in both the preclinical and clinical settings, making direct comparisons between studies challenging.

CONCLUSIONS

The prebiotic properties of lactulose have been known for over 60 years, and a wealth of data from studies published over the past 30 years shows that lactulose at a dose of 5–10 g/day exerts prebiotic effects. Nevertheless, lactulose is not widely used as a prebiotic. These studies have demonstrated the efficacy of low-dose lactulose in stimulating proliferation of *Bifidobacterium* and *Lactobacillus* spp., increasing beneficial SCFAs, especially acetate and (to a lesser degree) butyrate, reducing certain clostridia, and improving mineral absorption, while eliciting few adverse effects. Of note, the immune regulatory effects of acetate (the main SCFA produced by lactulose fermentation) may have important implications for regulating the inflammatory response, important for both controlling infections and reducing the risk of chronic inflammatory conditions, including osteoporosis, gout, and CRC. Furthermore, the ability of lactulose to enhance Ca absorption may have implications for enhancing bone density and bone health, which may be of particular clinical relevance for adolescents, postmenopausal women, and individuals at an advanced age. Further studies are required to establish whether the beneficial effects of lactulose can be seen in patients with various pathologies, and whether therapeutic or preventive use of lactulose may be beneficial in diseases such as osteoporosis and CRC.

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All authors have contributed substantially to the conception and design of the article, to the analysis and interpretation of the relevant data and literature, and to the drafting and critical revision of the content.

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Impact of Citrus Pulp or Inulin on Intestinal Microbiota and Metabolites, Barrier, and Immune Function of Weaned Piglets

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We investigated the use of citrus pulp (CP) as a novel prebiotic capable of exerting microbiota and immunomodulating capacities to alleviate weaning stress. Inulin (IN), a well-known prebiotic, was used for comparison. Hundred and 28 male weaned piglets of 21 days old were assigned to 32 pens of 4 piglets each. Piglets were assigned to one of the four treatments, i.e., control, IN supplemented at 0.2% (IN0.2%), and CP supplemented either at 0.2% (CP0.2%) or at 2% (CP2%). On d10–11 and d31–32 post-weaning, one pig per pen was euthanized for intestinal sampling to evaluate the growth performance, chyme characteristics, small intestinal morphology, colonic inflammatory response and barrier integrity, metabolite profiles [gas chromatography-mass spectrometry (GC-MS), and liquid chromatography-mass spectrometry (LC-MS)], and microbial populations. The IN treatment and the two CP treatments induced higher small intestinal villus height to crypt depth ratios in comparison with the control diet at both sampling times. All treatments decreased acidic goblet cell absolute counts in the crypts in comparison to the control diet of the duodenum on d10–11 and d31–32. The gene expression of β-defensin 2 was downregulated in colonic tissues following the IN and CP2% inclusion on d31–32. On d31–32, piglets fed with IN and CP0.2% showed lower mRNA levels of occludin and claudin-3, respectively. Not surprisingly, flavonoids were observed in the colon in the CP treatments. Increased colonic acetate proportions on d10–11, at the expense of branched-chain fatty acid (BCFA) levels, were observed following the CP2% supplementation compared to the control diet, inferring a reduction of proteolytic fermentation in the hindgut. The beneficial microbial community *Faecalibacterium* spp. was promoted in the colon of piglets fed with CP2% on d10–11 ($p = 0.04$; false discovery rate (FDR) non-significant) and on d31–32 ($p = 0.03$; FDR non-significant) in comparison with the control diet. Additionally, on d31–32, CP2% increased the relative abundance of *Megasphaera* spp. compared to control values ($p = 0.03$; FDR non-significant).

In conclusion, CP2% promoted the growth of beneficial bacterial communities in both post-weaning time points, modulating colonic fermentation patterns in the colon. The effects of CP supplementation were similar to those of IN and showed the potential as a beneficial feed supplement to alleviate weaning stress.

Keywords: citrus pulp, inulin, intestinal health, inflammation, barrier function, gut morphology, microbiota, metabolites

INTRODUCTION

In the pig industry, the selection for hyper-prolific sows has caused higher mortality and morbidity in early life due to low body weight at birth, and more competition for suckling milk (1). For mammals, lactation is essential for appropriate postnatal gut development, especially in pigs due to the lack of maternal transfer of immunity *via* the placenta. In pig production, there is a tendency for shortening the lactation period with the objective to increase sow productivity by reducing the estrus interval, which results in an early and abrupt transition for piglets from a milk-based diet to a solid-feed diet. Indeed, weaning is a critical event in swine production comprising physiological, social, nutritional, and environmental challenges that result in detrimental changes in gut morphology, physiology, immunology, and function (2, 3). Moreover, as the mucosal immune system is still immature and a stable commensal microbiota is not yet established (4), this period is often associated with disturbed microbiota and the proliferation of pathogens in the hindgut, resulting in post-weaning diarrhea (5, 6). In addition, it has been demonstrated that weaning is associated with pro-inflammatory events in the host hindgut (7) and the marked changes in intestinal histology and barrier integrity (8).

Weaning stress can have an impact on the intestinal wall architecture leading to villus atrophy and crypt hyperplasia, which further compromises the digestive, absorptive, and secretory capacities of the small intestine (9). Besides, weaning coincides with a disruption of the intestinal barrier function with a loss of integrity (10, 11); as well as a destabilization of the local immune system and mucosal inflammation (12, 13). The composition and diversity of the gut microbiota as well as its metabolic activities are highly influenced by an abrupt change of diet. For instance, a rapid decline in *Lactobacillus* spp. is observed during the weaning transition, while *Clostridium* spp., *Prevotella* spp., *Proteobacteriaceae*, and *E. coli* are increased (14). The shift of microbiota carries compositional and functional instabilities that often lead to dysbiosis, and consequently, to post-weaning disorders.

To overcome post-weaning losses and limit the use of antimicrobial compounds, dietary inclusion of functional ingredients and supplements remains an interesting strategy in pig production although their modes of action on gut health are still unclear (15–17) and chiefly depend on the specific composition. The functional activities of prebiotic ingredients include the stimulation of microbial fermentation, the proliferation of commensal bacterial communities, and the production of metabolites, such as short-chain fatty acids (SCFAs), attenuating hindgut protein fermentation (18), and conferring health-related benefits to the host (19, 20).

Citrus pulp (CP), including peels, internal tissues, and seeds, contains large amounts of soluble carbohydrates, including pectin (21, 22), which is extensively fermented by the hindgut microbiota (23, 24). Although CP has been primarily used in ruminant nutrition (21), it may also be considered as an alternative prebiotic in the weaned pigs (25–27). We hypothesized that the dried CP might have prebiotic activities similar to those of inulin (IN), a soluble fiber-rich fraction from the chicory sector (28), and a well-known feed supplement used in swine nutrition (29). Dietary IN has been shown to exert beneficial effects in the weaned piglets, including the stimulation of the immune system and the modulation of beneficial bacterial communities in the hindgut (29, 30). Therefore, the aim of the current study was to investigate the dose-dependent prebiotic potential of CP (CP0.2% and CP2%) next to IN (IN0.2%) in comparison with a basal control feed, devoid of health-modulating supplements, in terms of microbial, metabolomic, and immunomodulatory capacities during the post-weaning period.

MATERIALS AND METHODS

Experiment 1

Animals and Diets

All experimental procedures on piglets were in accordance with European and Belgian regulations concerning the care and use of animals for research purposes and were performed at the facilities of the Nuscience Group (Melle, Belgium) under the license number A04/357 granted by the ethical commission of the University of Ghent to perform animal experiments.

A total of 128 male Large White × Danish Landrace × Piétrain piglets weaned at 21 days and with an average weight of 5.2 ± 0.5 kg (mean ± SD) were obtained from a commercial farm (Flanders, Belgium). Only male piglets were considered in this study to limit sex disparity in immunological function and susceptibility (31). The animals were allocated to 32 pens of 4 littermates based on body weight and were fed *ad libitum* during a 5-day adaptation period with a commercially formulated creep feed diet (Babistar Flex©, Nuscience Group, Melle, Belgium). Piglets were housed in pens with fully slatted plastic floors and wire-mesh sides in a temperature-controlled animal house, including eight identical rooms (with four pens each). Each pen was equipped with a feeder and a drinking bowl. Following the 5-day adaptation period, piglets were assigned to one of the four treatments ($n = 8$ pens): a control diet, an IN diet (control

feed additionally supplemented with 0.2% of IN; Cosucra Group Warcoing SA, Warcoing, Belgium), and two CP diets (control feed additionally supplemented with either 0.2% or 2% of the dried CP (CP0.2/CP2%); Nuscience Group, Melle, Belgium). The dose of IN was chosen as suggested by the producer and based on the previous studies found in the literature that used 0.4–0.6% IN supplementation (32, 33). Moreover, Metzler-Zebeli et al. (34) showed that 0.2% IN was the median dose used in overall 43 studies, which was used in this study as a minimal effective dose. Therefore, the same dose was chosen for CP, and 10 times higher dose to reach similar amounts as described in few earlier studies (25–27). In each of the eight rooms, there was one pen per experimental diet. The basal diet was formulated to reach the nutritional requirements of animals following the NRC recommendations (**Table 1**). For the chemical composition of IN and CP, we refer to Uerlings et al. (35). Throughout the experiment, the pigs had *ad libitum* access to feed and water.

Six piglets facing too severe weight loss or with leg injuries after weaning had to be treated with antibiotics while one piglet suddenly died. Therefore, these were excluded from the experiment without affecting the number of experimental units, reaching eight pens per treatment for zootechnical performance parameters and eight piglets per treatment for the other parameters. A power analysis was performed using G*Power software to calculate the sample size required for the experiment.

TABLE 1 | Ingredient proportions of the control diet* and analyzed chemical composition.

INGREDIENTS (%)	
Barley	28.0
Corn heat treated	16.4
Wheat	15.0
Wheat heat treated	10.0
Soybean Danex	13.1
Soybean protein concentrate	4.0
Soybean meal	3.0
Soybean oil	1.5
DL-Methionine	0.24
L-Lysine	0.62
L-Threonine	0.29
L-Tryptophan	0.09
L-Valine	0.13
Salt	0.50
Monocalcium phosphate	0.73
Limestone	0.40
Premix**	6.0
NUTRIENT COMPOSITION	
Dry matter (%)	89.8
Crude protein (%)	16.9
Crude fat (%)	5.8
Crude ash (%)	4.6
Crude fiber (%)	4.7
Starch (%)	41.5
Sugars (%)	5.8
Calcium (%)	0.4
Phosphorus (%)	0.4
Sodium (%)	0.2
Zinc (%)	0.8
Manganese (%)	0.8
Iron (%)	2.8
Cupper (%)	1.0

IN, CP0.2%, and CP2% treatments were formulated accordingly as the small levels of inclusion of the tested ingredients did not modify the analyzed chemical composition of the diets.

***The premix contained vitamins, trace elements, flavoring compounds, enzymes, and MCFA, providing the following quantities per kg of diet: vitamin A, 15,000 IU; vitamin D3, 2,000 IU; vitamin E, 110 mg; vitamin K3, 3 mg; vitamin B1, 1.5 mg; vitamin B2, 5.4 mg; calcium D-pantothenate, 17.9 mg; vitamin B6, 2.9 mg; vitamin B12, 0.04 mg; nicotinamide, 30.3 mg; choline chloride, 750 mg; iron(II) sulfate monohydrate, 120 mg; copper(II) sulfate pentahydrate, 150 mg; zinc sulfate monohydrate, 75 mg; zinc chelate of glycine hydrate, 25 mg; manganese(II) oxide, 80 mg; calcium iodate, 1 mg; sodium selenite, 0.35 mg; endo-1,4-beta-glucanase, 250 TGU; endo-1,4-beta-xylanase, 560 TXU; 6-phytase, 1,000 FYT.*

Feed Chemical Analyses

Moisture, crude ash, crude fiber, crude protein, crude fat, sugar, and starch contents in the feed were measured using near-IR spectroscopy (Tango-R, Bruker Belgium N.V., Kontich, Belgium). Minerals and trace elements were analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES; Avio 500, PerkinElmer, Waltham, MA, USA) according to the EN15510 method.

Zootechnical Performances and Fecal Consistency

Feed intake and body weight gain were weekly recorded throughout the entire experiment ($n = 8$ pens per treatment). The diarrhea status of the piglets was assessed visually, by a single observer, with fecal scoring every 2 days using a scale going from 1 to 3 (1 = hard or soft dry pellets; 2 = soft wet-shaped pellets; and 3 = unshaped soft pellets and watery feces). Each score was given per pen ($n = 8$ pens per treatment) and was converted into a weekly occurrence.

Sampling of Intestinal Tissues and Contents

One pig per pen, with an average pen body weight, was euthanized on post-weaning days 10–11, i.e., 5–6 days after receiving the experimental diets, and d31–32 post-weaning, when piglets were 31–32 and 52–53 days of age, respectively ($n = 8$ animals per treatment). The sampling points were organized in 2 consecutive days with a randomized scheme and an equal distribution of animals from each experimental group to be euthanized per day. Anesthesia was applied by an intramuscular injection of a mix of Xylazine (Dopharma, Raamsdonkveer, the Netherlands) and Zoletil 100 (Virbac, Barneveld, the Netherlands) resulting in doses of 4 mg kg^{-1} BW of xylazine, 2 mg kg^{-1} BW of zolazepam, and 2 mg kg^{-1} BW of tilamine. After anesthesia, piglets were euthanized with an intracardiac injection of sodium pentobarbital (0.2 ml kg^{-1} BW; Release®, ECUPHAR NV/SA, Oostkamp, Belgium) and were immediately exsanguinated by the severance of the carotid arteries and jugular veins.

Tissue samples were collected from the duodenum (about 15 cm after the pyloric junction), jejunum (middle section of

the small intestine), and ileum (about 20 cm from the ileocecal valve), rinsed with a saline solution, and fixed in phosphate-buffered formalin (10%, pH 7.6) for 48 h prior to storage in 70% ethanol for histomorphometric measurements. Colonic tissues were rinsed with saline, snap-frozen, and stored at −80°C until the gene expression assay. Ileal, cecal, and colonic contents were collected. Ileal content was immediately stored on wet ice until viscosity measurements. Furthermore, ileal, cecal, and colonic contents were snap-frozen and stored at −80°C until further analyses of metabolites.

Viscosity Measurements

Soluble fibers are more extensively and rapidly fermented than insoluble polysaccharides due to their higher water-holding capacity, which increases the viscosity of the digested and allows bacteria to easily penetrate the matrix (36). Therefore, the viscosity of the ileal digesta was determined. Ileal digesta (n = 8 animals per treatment) collected on d31–32 were centrifuged for 15 min at 21,000 *g*, and the viscosity of a 500-µl supernatant was measured with a CP40 cone and a constant shear rate of 450 s^{-1} (Brookfield DV II+ viscometer; Brookfield, Middleboro, MA, USA). Viscosity measurements could not be achieved on d10–11 because of the lack of sufficient ileal content.

Histomorphology Metrics

Tissues from the duodenum and jejunum (n = 8 animals per treatment/sampling time) collected on d10–11 and d31–32, were embedded in paraffin wax, cut at a 5-µm thickness with a microtome using Thermo MX35 Ultra blades (Thermo Fisher Scientific, Waltham, MA, USA), and stained with Alcian Blue-Periodic Acid Schiff. Twenty well-oriented villus-crypt units were selected on each slide to determine villus height and width as well as crypt depth (µm) by 10-fold microscopy (Olympus Corporation, Tokyo, Japan). The number of acidic and neutral goblet cells (count per crypt), the density in total goblet cells (count per 100 µm of the crypt), the thickness of the *muscularis mucosae* and *tela submucosa,* and the *tunica muscularis* thickness (µm) were determined.

Gene Expression Assay in Colonic Tissue

Total RNA from colonic tissues (n = 8 animals per treatment/sampling time) collected on d10–11 and d31–32 was extracted using the ReliaPrep RNA Tissue Miniprep System (Promega Corporation, Madison, WI, USA) according to the protocol of the manufacturer. RNA concentration and quality were determined by Nanodrop dosage (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel (1%), respectively. The extracted RNA (75 ng) was converted into cDNA using the Reverse Transcription Master Mix (Fluidigm Corporation, South San Francisco, CA, USA). Samples were thereafter pre-amplified according to the PreAmp MasterMix instructions provided by the manufacturer (Fluidigm Corporation, South San Francisco, CA, USA), followed by an exonuclease I treatment (New England Biolabs, Ipswich, MA, USA).

High-throughput quantitative PCR (qPCR) was performed as previously described (35) with intron-spanning primer pairs (**Supplementary Table 1**) designed using Primer-BLAST (NCBI) and validated through agarose gel electrophoresis and through melting curves. High-throughput qPCR was performed in 48 × 48 dynamic array-integrated fluidic circuits (Fluidigm Corporation, South San Francisco, CA, USA) following the protocol: 60 s at 95°C, followed by 30 cycles (5 s at 96°C and 20 s at 60°C). Quantification cycles (*Cq*) were acquired using the Fluidigm real-time PCR analysis software 3.0.2 (Fluidigm Corporation, South San Francisco, CA, USA).

First, all housekeeping genes were evaluated, and the four most stable genes between treatments were determined by NormFinder (37). The selected four reference genes consisted of hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), ribosomal protein L4 (*RPL4*), TATA-box binding protein (*TBP*), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*). For each target and housekeeping gene analyzed, the relative gene expression level was calculated using the Pfaffl method (38), and the geometrical mean of the relative expression of the four housekeeping genes was used to normalize all samples.

High-Performance Liquid Chromatography Metabolite Profiling of Ileal, Cecal, and Colonic Contents

Ileal, cecal, and colonic contents collected on d10–11 and d31–32 of the experiment (n = 8 animals per treatment/sampling time/intestinal segment) were six-fold diluted in ultrapure water prior to metabolite determination by high-performance liquid chromatography (HPLC). Intermediate metabolites (lactate, pyruvate, succinate, and formate), SCFAs (acetate, propionate, and butyrate), and branched-chain fatty acids (BCFAs; *i*-butyrate, *i*-valerate, and valerate) were analyzed by isocratic HPLC using an Alliance System e2695 (Waters Corporation, Milford, MA, USA) with an Aminex HPx-87H column (BioRad, Hercules, CA, USA) combined with a UV detector (210 nm), with H_2SO_4 (5 mM) as a mobile phase at an eluent flow of 0.6 ml min^{-1} and with a temperature of 60°C. Each peak was integrated using the Empower 3 software (Waters Corporation, Milford, MA, USA) and quantified using an external standard calibration. Although valerate is not a BCFA *per se*, the organic acid is usually classified as a metabolite from the proteolytic fermentation and is therefore included within the BCFA group. Intermediate metabolites and the sum of SCFAs were expressed in mg g^{-1} of fresh content. Acetate, propionate, butyrate, and BCFA amounts were expressed as a ratio (%) of the sum of SCFAs (39). As there were only differences in SCFA and BCFA in the colonic content, these samples were chosen for further metabolome analyses by gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS).

GC-MS Metabolomic Profiling of Colonic Contents

The GC-MS analysis was performed at the VIB metabolomics core Ghent (Ghent, Belgium). Samples were dissolved in 1 ml of methanol and divided into two aliquots of 400 µl each. For each sample, one of the aliquots was evaporated to dryness under vacuum, and the obtained residue was derivatized by adding 10 µl of pyridine and 50 µl of N-Methyl-N-(trimethylsilyl)-trifluoroacetamide (Sigma-Aldrich, Saint Louis, MO, USA). The

GC-MS analysis was carried out using a 7,890B GC system equipped with a 7,693A Automatic Liquid Sampler and a 7,250 Accurate-Mass Quadrupole Time-of-Flight (QTOF) MS system (Agilent Technologies, Santa Clara, CA, USA). About 1 μl of the sample was injected in a split-less mode with an injector port set to 280°C. Separation was achieved with a VF-5ms column (30 m × 0.25 mm × 0.25 μm; Varian CP9013; Agilent Technologies, Santa Clara, CA, USA) with helium as a carrier gas at a constant flow of 1.2 ml/min. The oven was held at 80°C for 1 min, ramped to 280°C at 5°C/min, held at 280°C for 5 min, ramped to 320°C at 20°C/min, held at 320°C for 5 min, and finally cooled to 80°C at 50°C/min at the end of the run. The MSD transfer line was set to 280°C, and the electron ionization energy was 70 eV. Full EI-MS spectra were recorded between m/z 50–800 at a resolution of >25,000.

Data processing was done in MassHunter Profinder (Agilent Technologies, Santa Clara, CA, USA) and includes feature extraction, combined with a chromatographic alignment across multiple data files. The appearance of both false positive and false negative features is minimized by "binning" the features in a chromatographic time domain. The NIST 17 Mass Spectral Library was accessed to screen for the spectra that matched the compounds present in the GC-MS chromatogram.

LC-MS Metabolomics Profiling of Colonic Contents

The LC-MS analysis was performed at the VIB metabolomics core Ghent (Ghent, Belgium). Samples were dissolved in 1 ml of methanol and divided into two aliquots of 400 μl each. For each sample, one of the aliquots was evaporated to dryness under vacuum, and the obtained residue was reconstituted in cyclohexane/water (1:1 v/v) (Sigma-Aldrich, Saint Louis, MO, USA). The extract in the water phase was used for the following ultra-high-performance liquid chromatography (UHPLC) analysis. The latter was performed on an ACQUITY UPLC I-Class system (Waters Corporation, Milford, MA, USA) consisting of a binary pump, a vacuum degasser, an autosampler, and a column oven. Chromatographic separation was carried out on an ACQUITY UPLC BEH C18 (150 × 2.1 mm, 1.7 μm) column (Waters Corporation, Milford, MA, USA) at 40°C. A gradient of two buffers was used: buffer A (99:1:0.1 water:acetonitrile:formic acid, pH 3) and buffer B (99:1:0.1 acetonitrile:water:formic acid, pH 3), as follows: 99% A for 0.1 min decreased to 50% A in 30 min, decreased to 30% in 5 min, and decreased to 0% in 2 min. The flow rate was set to 0.35 ml min^{-1}, and the injection volume was 10 μl. The UHPLC system was coupled to a Vion IMS QTOF hybrid mass spectrometer (Waters Corporation, Milford, MA, USA). The LockSpray ion source was operated in negative electrospray ionization (ESI) mode under the following specific conditions: capillary voltage, 2.5 kV; reference capillary voltage, 3 kV; cone voltage, 40 V; source offset, 50 V; source temperature, 120°C; desolvation gas temperature, 600°C; desolvation gas flow, 800 L h^{-1}; and cone gas flow, 50 L h^{-1}. Mass range was set from 50 to 1,000 Da. The collision energy for full HDMSe was set at 6 eV (low energy) and ramped from 20 to 70 eV (high energy), the intelligent data capture intensity threshold was set at 5. Nitrogen (greater than 99.5%) was employed as desolvation and cone gas. Leucin-enkephalin (250 pg μl^{-1} solubilized in water:acetonitrile 1:1 [v/v], with 0.1% formic acid) was used for the lock mass calibration, with scanning every 2 min at a scan time of 0.1 s.

Profile data were recorded through a UNIFI Scientific Information System (Waters Corporation, Milford, MA, USA). ESI interface was employed in both positive and negative modes. Data processing was performed with Progenesis QI software version 2.4 (Waters Corporation, Milford, MA, USA). The in-house Mass Spectral Library (PhytoComp) and external spectral libraries (MONA; https://mona.fiehnlab.ucdavis.edu/) were used to screen for the spectra that match the compounds present in the LC-MS chromatogram.

DNA Extraction, Sequencing, and Bioinformatics of Colonic Contents

DNA from colonic contents collected on d10–11 and d31–32 of the experiment ($n = 8$ animals per treatment/sampling time) was extracted using the QIAamp PowerFaecal Pro DNA kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. The DNA concentration and quality were determined by Nanodrop dosage (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel (1%), respectively. Following DNA extraction, 16S rRNA gene sequencing was performed by DNAVision (Gosselies, Belgium), using the Illumina MiSeq technologies (2 × 250 nt). The F-primer (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGC AG-3′) and the R-primer (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′) were used to amplify the hypervariable regions V3-V4 according to the 16S Metagenomic Sequencing Library Preparation protocol from Illumina.

Quantitative Insights into Microbial Ecology (QIIME) software package (version 1.9.0) was used for operational taxonomic unit (OTU) clustering, with an identity cutoff of 97% by comparison to the Greengenes reference database 13.8. Beta diversity was measured using weighted UniFrac distance metrics with principal coordinate analysis (PCoA) using QIIME. The ADONIS method was used to determine whether communities differed significantly between groups of samples. Microbiota results were analyzed per time point using a Kruskall–Wallis test with the treatment as a fixed factor. The false discovery rate (FDR) correction was used to calculate the adjusted p-values. Microbial alpha diversity metrics, including Chao1, phylogenetic diversity whole tree, observed OTU, and Shannon indexes, were calculated using QIIME. Raw sequences have been uploaded to the European Nucleotide Archive database (project number PRJEB38284). Pearson's correlation coefficients between metabolites and microbiota communities were also calculated.

Experiment 2

Animals

A second experiment was run in Gembloux Agro-Bio Tech, University of Liège, in Gembloux (Belgium) to determine intestinal permeability. All experimental procedures on piglets were in accordance with European and Belgian regulations concerning the care and use of animals for research purposes

and were approved by the Animal Ethical Committee of Liège University, Belgium (protocol number: 1,860).

A total of 32 male Landrace × Piétrain piglets weaned at 21 days and with an average weight of 5.9 ± 0.6 kg (mean ± SD) were obtained from the Walloon Agricultural Research Center in Gembloux (Belgium). The animals were allocated to 16 pens of 2 littermates based on body weight. Piglets were housed in a temperature-controlled animal house with slatted floors and with a feeder and a drinking nipple per pen. Following the 5-day adaptation period with the commercial creep feed diet, piglets were assigned to one of the four treatments ($n = 4$ pens): a control diet, an IN diet at 0.2% (IN), and two CP diets (CP0.2/CP2%). Throughout the experiment, the pigs had *ad libitum* access to feed and water.

In vivo Intestinal Permeability

Intestinal permeability was assessed *in vivo* after 7 days of treatment with a sugar absorption test. Pigs ($n = 8$ per treatment) were initially fasted overnight and were subsequently administered with an oral dose of 5 ml kg^{-1} of sugar solution, containing D-xylose (100 mg kg^{-1}; VWR International, Oud-Heverlee, Belgium) dissolved in water. One hour after oral administration, a 5-ml blood sample was collected from the jugular vein into gel and clot activator vacuum tubes (VWR International, Oud-Heverlee, Belgium). After centrifugation (10 min, 2,000 g at 4°C), serum samples were stored at −20°C until the analysis of serum concentration of D-xylose as a marker of intestinal absorptive capacities and mucosal integrity.

Determination of D-Xylose in Serum

Serum D-xylose was determined as described by Eberts et al. (40). Briefly, D-xylose standard solutions were prepared by dissolving D-xylose in saturated benzoic acid to reach 0, 50, 100, 200, 400, and 800 mg L^{-1} concentrations. The phloroglucinol color reagent solution was prepared with 0.5 g of phloroglucinol, 100 ml of glacial acetic acid, and 10 ml of concentrated hydrochloric acid (all from Sigma-Aldrich Co., St Louis, MO, USA).

The standard and serum samples (50 µl) were added to 5 ml of phloroglucinol color reagent solution, were heated at 100°C for 4 min, and were allowed to cool down in a water bath at room temperature. The absorbance was determined at 570 nm in a spectrophotometer (VICTOR plate reader, PerkinElmer, Waltham, MA, USA). The standard solution of 0 mg L^{-1} D-xylose was considered as blank, and xylose-free serum was used to obtain the net absorptive concentrations.

Statistical Analyses

Zootechnical performance was analyzed with mixed procedures, the treatment being a fixed factor, the pen being a random factor, and using the initial body weight as a covariate. Fecal scoring was also analyzed using a mixed procedure with the treatment as a fixed factor and the pen as a random factor. Viscosity measurements and serum xylose concentrations were subjected to generalized linear model (GLM) procedures using one fixed criterion of classification (treatment). Microbiota and metabolomic results and gene expression data were analyzed per time point, using the treatment as a fixed factor in the GLM procedure. The FDR correction was used to calculate the adjusted *p*-values for the gene expression and microbiota and metabolomic results (41). The histomorphometrical data were analyzed per time point, using the treatment as a fixed factor and the slaughtering weight as a covariate in the GLM procedure. Gene expression and histomorphometrical data were analyzed per time point as we chose to focus on the treatment effect rather than the age effect, which was not considered as the main aim of this research. The comparison of means was evaluated by *post hoc* Tukey's multiple comparison honestly significant difference (HSD). For the metabolomic data, ANOVA, heatmaps, and PCA plots were generated using the online tool MetaboAnalyst 4.0. The *P*-values <0.05, $p < 0.01$, and $p < 0.001$ were considered as statistically significant, highly significant, and very highly significant. All statistical analyses were performed using SAS 9.4 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Experiment 1

Zootechnical Performances

Weekly body weight gain and feed intake remained unaffected by diets ($p > 0.05$). Piglets fed with CP2% displayed a significantly higher feed conversion ratio (1.66 ± 0.05) in comparison to their control counterparts (1.45 ± 0.06) after 1 week of treatment ($p < 0.05$), which was no longer observed during the 3 following weeks and for the entire experimental period (**Supplementary Table 2**). Piglet receiving CP2% demonstrated significantly lower proportions of score 1 feces, dry pellets, during the 2nd week of treatment (**Figure 1A**). No other significant differences were observed for weeks 1, 3, and 4 (data not shown) or for the entire experimental period (**Figure 1B**).

The Viscosity of the Ileal Digesta

The CP2% diet (2.69 ± 0.36 cP) induced a significant rise in ileal chyme viscosity in comparison with control (1.48 ± 0.06 cP) and CP0.2% (1.48 ± 0.09 cP) treatments on d31–32 ($p < 0.0001$), while IN-treated pigs (2.03 ± 0.03 cP) demonstrated intermediate values.

Small Intestinal Histomorphology

At d10–11, histological measurements showed a significantly reduced crypt depth with increased VH:CD ratio due to the treatment with CP and IN in all the studied segments of the intestine, including the duodenum, jejunum, and ileum (**Table 2**). The measurements at d31–32 showed a decrease in crypt depth in the duodenum ($p < 0.05$) and ileum ($0.05 < p < 0.1$) and a significant increase of VH:CD ratio due to the treatment in the duodenum, jejunum, and ileum ($p < 0.05$).

Inulin-treated pigs displayed the thickest and the thinnest duodenal *muscularis mucosae* and *tela submucosa* layers on d10–11 and d31–32, respectively, reaching statistical trends ($p < 0.1$; **Supplementary Table 3**). No further differences were observed in other histomorphometrical measurements.

Acidic goblet cells showed a significant decrease in counts due to the treatment at days 10–11 in the duodenum and ileum (*p*

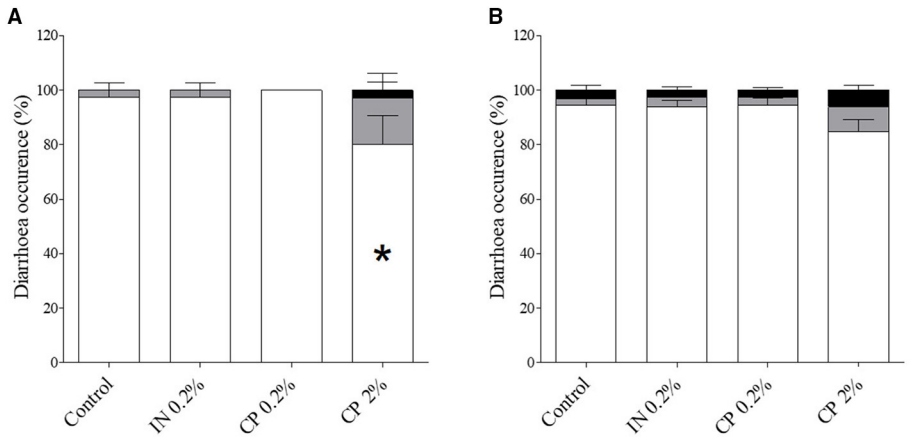

FIGURE 1 | Diarrhea occurrence in pigs after feeding inulin (IN) (IN0.2%) and citrus pulp (CP) (CP0.2% and CP2%) from post-weaning day 5, after 2 weeks **(A)** and for the entire 4-week experiment **(B)**. □ *Score 1* (hard or soft dry pellets); ▒ *Score 2* (soft wet-shaped pellets); ■ Score 3 (unshaped soft pellets and watery feces). Mean values ($n = 8$ pens) ± SEM. Symbols (*) within bars indicate significant differences between groups ($p < 0.05$).

< 0.05) (**Table 2**). Total goblet cell counts were also significantly affected by the treatment in the ileum section of the intestine ($p < 0.05$), while the duodenum showed a tendency. At the sampling times of 31–32 days, the acidic goblet cell count was significantly reduced due to the treatment in the jejunum section ($p < 0.05$), while it remained unaffected in the other sections.

Gene Expression in Colonic Tissue

Apoptosis-related genes in a colonic tissue (**Figure 2**) showed downregulation of caspase 1 (*CASP1*) in the IN treatment compared to control pigs on d31–32 (**Figure 2B**). None of the 11 genes involved in signaling pathways of inflammation were altered following IN or CP supplementation, neither on d10–11 nor on d31–32, as seen in **Supplementary Figure 1** ($p > 0.05$). The inflammation target gene (**Figure 3**) β-defensin 2 expression was downregulated in the colonic tissue of CP2%- and IN-fed piglets in comparison to control piglets on d31–32 (**Figure 3B**). Barrier integrity genes (**Figure 4**) showed an upregulation of tricellulin (*MARVELD2*) on d10–11 in CP2%-treated pigs, which was significantly different from that in the CP0.2% treatment (**Figure 4A**). On d31–32, claudin-3 and mucin 2 (*MUC2*) mRNA levels were lower with CP 0.2% in comparison to the control diet. At the same time, IN-fed pigs showed decreased expressions of *MUC2* and occludin compared to their control counterparts (**Figure 4B**). The FDR correction did not reveal any difference between diets for any of the genes.

SCFA and BCFA Profiles in the Ileal, Cecal, and Colonic Contents

The ileal and cecal SCFA and BCFA profiles remained unaffected by treatments, regardless of the sampling time (**Supplementary Table 4**). On d10–11, piglets receiving the CP2% diet had significantly lower colonic BCFA proportions in comparison to their control counterparts ($p < 0.01$; **Table 3**). At that time, significantly higher colonic acetate ratios were found with the CP2% treatment in comparison to the other treatments ($p < 0.01$; **Table 3**). At the same time, colonic lactate levels significantly increased following IN supplementation in comparison to CP0.2% and CP2% treatments ($p < 0.05$; **Table 3**). On d31–32, the CP2% diet tended to induce lower colonic propionate proportions, reaching a statistical trend ($p < 0.1$; **Table 3**).

Metabolomic Profile in the Colonic Content

The top significant features obtained with the LC-MS results are shown in **Table 4** and can be visualized in **Figure 5**. The ESI+ list showed 12 features at d10–11 and 11 features at d31–32. The ESI– list showed 14 features at d10–11 and 14 at d31–32, which mostly include flavonoids such as hesperidin, neohesperidin, naritin, and naringenin.

Moreover, LC-MS resulted in an ESI– list containing 89 significant feature ions between the treatments at d10–11, while at d31–32 there were 100 features significantly different at an FDR < 0.05 and p-value < 0.05 (data not shown). Eighty-three features were in common between both time points. For an ESI+ list, at d10–11, there were 114 features significantly different, while at d31–32, 127 features were significantly different between treatments, at an FDR<0.05 and p-value <0.05. For this list, 104 features were in common between both time points (data not shown).

For all the colonic content samples analyzed, GC-MS resulted in the detection of 329 compounds. The list of the top 10 identified compounds and heatmaps obtained at d10–11 and d31–32 that had a p-value < 0.05 for the treatment effect are presented in **Table 5** and visualized as (**Supplementary Figure 2**). None of them were significantly different between treatments after the FDR correction. Nonetheless, some of these identified compounds, such as octodecenoic acid, indole-acetic acid (IAA), and pipecolic acid, were interesting.

TABLE 2 | Histomorphometrical measurements in the duodenum, jejunum, and ileum intestinal segments after feeding inulin (IN) (IN0.2%) and citrus pulp (CP) (CP0.2% and CP2%) from post-weaning day 5 onward, at sampling times on d10–11 and d31–32 post-weaning.

		Control	IN0.2%	CP0.2%	CP2%	*P*-value
d10–11						
Villus height (μm)	Duodenum	436 ± 11	479 ± 19	430 ± 15	443 ± 14	0.1226
	Jejunum	448 ± 11	484 ± 17	504 ± 19	479 ± 17	0.13
	Ileum	388 ± 9	413 ± 13	411 ± 10	401 ± 11	0.3518
Villus width (μm)	Duodenum	127 ± 6	124 ± 7	117 ± 2	123 ± 6	0.6014
	Jejunum	167 ± 47	118 ± 4	115 ± 3	111 ± 3	0.3321
	Ileum	113 ± 1	108 ± 4	108 ± 3	111 ± 3	0.5098
Crypt depth (μm)	Duodenum	378^{a} ± 13	312^{b} ± 9	306^{b} ± 9	320^{b} ± 11	**0.0001**
	Jejunum	285^{a} ± 7	251^{b} ± 7	251^{b} ± 5	251^{b} ± 7	**0.0012**
	Ileum	245^{a} ± 8	223^{ab} ± 7	216^{b} ± 4	213^{b} ± 5	**0.0047**
VH:CD ratio*	Duodenum	1.17^{b} ± 0.02	1.57^{a} ± 0.07	1.44^{a} ± 0.07	1.42^{a} ± 0.06	**0.0002**
	Jejunum	1.60^{b} ± 0.03	1.96^{a} ± 0.06	2.06^{a} ± 0.1	1.94^{a} ± 0.05	**0.0002**
	Ileum	245^{a} ± 8	223^{ab} ± 7	216^{b} ± 4	213^{b} ± 5	**0.0047**
Neutral goblet cell count (count per crypt)	Duodenum	3.5 ± 2.5	0.6 ± 0.2	0.9 ± 2	1.3 ± 0.3	0.3625
	Jejunum	1.7 ± 0.5	1.1 ± 0.3	0.6 ± 0.2	1.2 ± 0.4	0.2862
	Ileum	0.9 ± 0.2	0.8 ± 0.2	0.7 ± 0.2	0.5 ± 0.1	0.3562
Acidic goblet cell count (count per crypt)	Duodenum	18.6^{a} ± 1.5	10.7^{b} ± 1.1	12.5^{b} ± 1	12.7^{b} ± 1.6	**0.0013**
	Jejunum	11.8 ± 1.1	9.8 ± 0.9	10.3 ± 0.9	8.9 ± 0.9	0.2134
	Ileum	15.9^{a} ± 0.5	11.9^{b} ± 0.8	10.8^{b} ± 1.2	11.4^{b} ± 1	**0.0019**
Total goblet cell density (count per 100 μm crypt)	Duodenum	5.7 ± 0.8	3.6 ± 0.3	4.4 ± 0.3	4.3 ± 0.5	0.0576
	Jejunum	4.8 ± 0.4	4.3 ± 0.3	4.4 ± 0.4	4 ± 0.4	0.5841
	Ileum	6.9^{a} ± 0.2	5.7^{ab} ± 0.3	5.3^{b} ± 0.5	5.6^{ab} ± 0.4	**0.0255**
d31–32						
Villus height (μm)	Duodenum	563 ± 20	604 ± 23	575 ± 25	606 ± 24	0.4808
	Jejunum	540 ± 17	529 ± 11	572 ± 19	545 ± 19	0.3334
	Ileum	417 ± 13	460 ± 25	487 ± 17	456 ± 25	0.1519
Villus width (μm)	Duodenum	145 ± 5	145 ± 12	137 ± 3	141 ± 5	0.856
	Jejunum	133 ± 4	123 ± 3	127 ± 2	123 ± 2	0.085
	Ileum	125 ± 3	128 ± 3	128 ± 3	131 ± 7	0.7975
Crypt depth (μm)	Duodenum	375^{a} ± 8	334^{b} ± 13	326^{b} ± 6	338^{ab} ± 10	**0.0058**
	Jejunum	313 ± 8	275 ± 7	289 ± 8	304 ± 33	0.4634
	Ileum	248 ± 4	231 ± 7	241 ± 7	224 ± 8	0.0956
VH:CD ratio*	Duodenum	1.53^{b} ± 0.03	1.86^{a} ± 0.05	1.80^{a} ± 0.07	1.83^{a} ± 0.06	**0.0009**
	Jejunum	1.75^{b} ± 0.04	1.96^{ab} ± 0.05	2.01^{a} ± 0.08	2.03^{a} ± 0.06	**0.0083**
	Ileum	1.71^{b} ± 0.06	2.02^{a} ± 0.07	2.06^{a} ± 0.08	2.07^{a} ± 0.08	**0.0046**
Neutral goblet cell count (count per crypt)	Duodenum	1.1 ± 0.3	0.8 ± 0.2	0.8 ± 0.2	0.6 ± 0.2	0.4287
	Jejunum	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.8297
	Ileum	0.4 ± 0.1	0.8 ± 0.3	0.7 ± 0.2	0.6 ± 0.2	0.438
Acidic goblet cell count (count per crypt)	Duodenum	16.6 ± 1.6	15.9 ± 1.8	13.3 ± 1.2	14.4 ± 1.6	0.438
	Jejunum	13.8^{a} ± 1.2	10.2^{b} ± 0.6	11.2^{ab} ± 0.8	10.3^{b} ± 0.9	**0.0285**
	Ileum	11.8 ± 1	10.5 ± 0.7	10.8 ± 0.9	11.4 ± 1.2	0.7514
Total goblet cell density (count per 100 μm crypt)	Duodenum	4.7 ± 0.5	5 ± 0.5	4.3 ± 0.4	4.5 ± 0.5	0.7507
	Jejunum	4.4 ± 0.3	3.8 ± 0.3	3.9 ± 0.3	3.6 ± 0.4	0.3624
	Ileum	4.9 ± 0.4	4.9 ± 0.2	4.7 ± 0.3	5.3 ± 0.5	0.6448

*****VH:CD, villus height to crypt depth ratio.*

Mean values ± SEM ($n = 8$ animals).

[a,b]Mean values within a row with unlike superscript letters are significantly different ($p < 0.05$).

The P-values <0.05, $p < 0.01$, and $p < 0.001$ were considered as statistically significant, highly significant, and very highly significant.

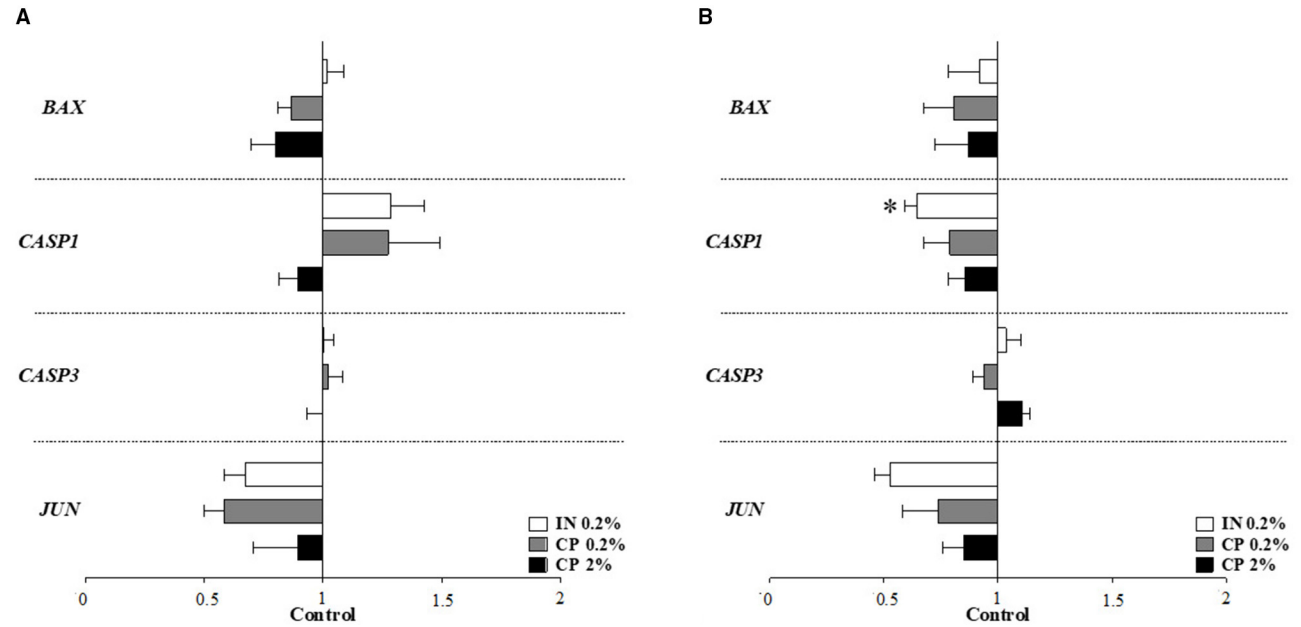

FIGURE 2 | Apoptosis-related target gene expression in the colonic tissue of pigs after feeding IN (IN0.2%) and CP (CP0.2% and CP2%) from post-weaning day 5, at sampling times on d10–11 **(A)** and d31–32 **(B)** post-weaning. BAX, BCL2-associated X protein; CASP, caspase; JUN, AP-1 transcription factor subunit. Figures display the % of the difference in comparison to the control treatment, considered as 1. Mean values ($n = 8$ animals) ± SEM. Symbol (*) denotes a significant difference between the treatments and the control ($p < 0.05$).

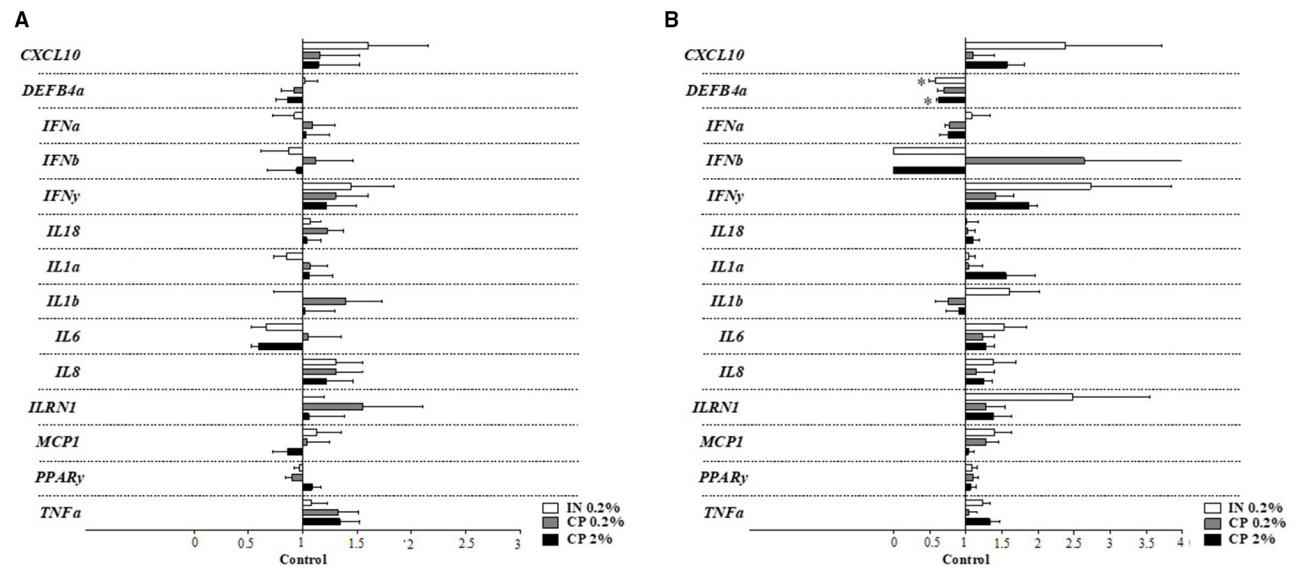

FIGURE 3 | Inflammatory target gene expression in the colonic tissue of pigs after feeding IN (IN0.2%) and CP (CP0.2% and CP2%) from post-weaning day 5, at sampling times on d10–11 **(A)** and d31–32 **(B)** post-weaning. CXCL10, C-X-C motif chemokine 10; DEFβ, defensin beta; IFN, interferon; IL, interleukin; ILRN1, interleukin-1 receptor antagonist; MCP-1, monocyte chemo attractant protein 1; PPARγ, peroxisome proliferator-activated receptor gamma; TNFα, tumor necrosis factor alpha. Figures display the % of the difference in comparison to the control treatment, considered as 1. Mean values ($n = 8$ animals) ± SEM. Symbol (*) denotes a significant difference between the treatments and the control ($p < 0.05$).

Microbiota in the Colonic Content

On d10–11, the observed OTU and phylogenetic diversity whole tree indexes were significantly impacted by treatments, whereas no significant differences were found for the Chao1 and Shannon indexes (**Table 6**), neither on d10–11 nor on d31–32. On d10–11, according to both the

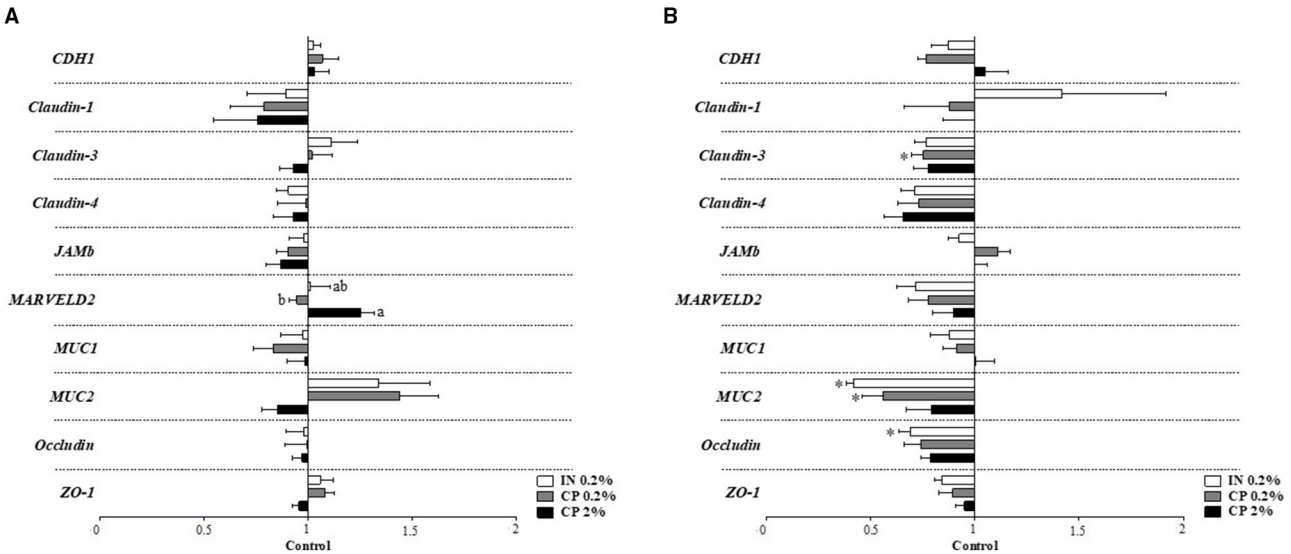

FIGURE 4 | Barrier-integrity target gene expression in the colonic tissue of pigs after feeding IN (IN0.2%) and CP (CP0.2% and CP2%) from post-weaning day 5, at sampling times on d10–11 **(A)** and d31–32 **(B)** post-weaning. CDH1, E-cadherin; JAM, junctional adhesion molecule; MARVELD2, tricellulin; MUC, mucin; ZO-1, zonula occludens-1. Figures display the % of the difference in comparison to the control treatment, considered as 1. Mean values ($n = 8$ animals) ± SEM. ^{a,b}Different superscript letters denote a significant difference between IN, CP0.2%, and CP2% ($p < 0.05$). Symbol (*) denotes a significant difference between the treatments and the control ($p < 0.05$).

observed OTU and phylogenetic diversity whole tree indexes, higher alpha diversity was found with the IN treatment in comparison to the CP0.2% treatment ($p < 0.05$). Concerning the observed OTU index, the IN treatment was also significantly higher than the control treatment ($p < 0.05$).

Sequencing of the 16S rRNA genes in colonic digesta produced a total of 3,900,165 quality-filtered sequences, with a mean number of 60,940 ± 29,645 reads (mean ± SD) per sample. Communities did not significantly differ between treatments, and no visual clustering could be detected on the weighted UniFrac PCoA plots, neither on d10–11 nor on d31–32 of the experiment (data not shown).

Colonic microbiota composition with a relative abundance of at least 1% was included in **Table 7**. At the first sampling time (d10–11), the main phyla observed were *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*; only two genera significantly differed between treatments, which disappeared after the FDR correction. The relative abundance of *Faecalibacterium* spp. was significantly increased in the colon of piglets fed with CP2% in comparison to the other diets. The colonic abundance in *Anaerovibrio* spp. was greater with IN and CP0.2% pigs compared to control pigs. When extending the analysis to microbiota communities with a relative abundance over 0.1% of the total colonic microbiota (**Supplementary Table 5**), six genera significantly differed at this sampling time although the FDR correction did not reveal any difference. The population of *Ruminococcus* spp. was lower in the colon of CP0.2%- and IN-fed piglets in comparison to the CP2%-supplemented piglets. The inclusion of CP2% induced the highest abundances of *Lachnospira* spp. (followed by CP0.2%) and unclassified *Peptostreptococcaceae*. Unclassified *p-2534-18B5* (followed by CP0.2%) and unclassified *Enterobacteriaceae* levels were significantly increased under the IN treatment.

On the second sampling time (d31–32), colonic bacterial sequences present over 1% of the total microbiota predominantly included the *Firmicutes*, *Bacteroidetes*, *Cyanobacteria*, and *Proteobacteria* phyla (**Table 7**). The abundance in colonic *Cyanobacteria* was significantly affected by treatments with control piglets, showing greater proportions of the phylum than CP2%- and IN-fed piglets. Five genera significantly differed in the colon of pigs administered with the treatments on d31–32, considering a relative abundance of over 1% of the total microbiota (**Table 7**). The FDR correction did not reveal any difference between diets except for the *Cyanobacteria* phylum and unclassified *2YS2* ($p < 0.05$). Unclassified *2YS2* belonging to the *Cyanobacteria* phylum was significantly lower with CP2% and IN treatments compared to the control feed. Decreased levels of unclassified *Lachnospiraceae* were found in the colon of piglets offered CP2% in comparison to the control and IN-fed piglets. CP2% induced a higher abundance of *Faecalibacterium* spp., significantly differing from control and CP0.2% treatments. The population of *Megasphaera* spp. was increased by CP2% compared to the other treatments. The *Bulleidia* spp. the genus was present in higher proportions in the colon of IN and CP0.2%-fed piglets compared to the other two treatments. Considering genera present with a relative abundance of over 0.1% of the total colonic microbiota, five genera significantly differed between treatments although the FDR correction did

TABLE 3 | Metabolite concentrations in the colonic content of piglets after feeding IN (IN0.2%) and CP (CP0.2% and CP2%) from post-weaning day 5 onward, at sampling times on d10–11 and d31–32 post-weaning.

Sampling time	Treatment	Lactate (mg g^{-1} OM)	Pyruvate (mg g^{-1} OM)	Succinate (mg g^{-1} OM)	Formate (mg g^{-1} OM)	SCFAs (mg g^{-1} OM)	Acetate (%)	Propionate (%)	Butyrate (%)	BCFAs (%)
d10–11	Control	$0.3^{ab} \pm 0.11$	0.01 ± 0.01	0.02 ± 0.01	0 ± 0	8.33 ± 0.4	$49.23^{b} \pm 1.44$	30.09 ± 1.52	17.01 ± 1.35	$3.67^{a} \pm 0.81$
	IN0.2%	$0.72^{a} \pm 0.21$	0.01 ± 0	0.03 ± 0.02	0 ± 0	7.85 ± 0.29	$49.58^{b} \pm 1.26$	29.83 ± 0.76	18.02 ± 1.21	$2.56^{ab} \pm 0.7$
	CP0.2%	$0.18^{b} \pm 0.07$	0 ± 0	0 ± 0	0 ± 0	8.6 ± 0.22	$49.13^{b} \pm 1.3$	31.38 ± 1.51	17.57 ± 1.53	$1.92^{ab} \pm 0.42$
	CP2%	$0.22^{b} \pm 0.08$	0.01 ± 0.01	0.01 ± 0	0 ± 0	8.31 ± 0.38	$56.20^{a} \pm 1.48$	28.63 ± 0.78	14.53 ± 0.96	$0.64^{b} \pm 0.26$
	P-value treatment	0.0229	0.4509	0.2343	0.4074	0.4641	**0.0022**	0.4634	0.2411	**0.0098**
d31–32	Control	0.28 ± 0.15	0 ± 0	0 ± 0	0 ± 0	7.92 ± 0.44	48.17 ± 1.3	29.47 ± 1.5	20.54 ± 1.29	1.82 ± 0.75
	IN0.2%	0.51 ± 0.45	0.01 ± 0.01	0 ± 0	0 ± 0	7.67 ± 0.49	48.99 ± 1.95	29.93 ± 1.12	20.88 ± 1.76	0.2 ± 0.17
	CP0.2%	0.16 ± 0.1	0 ± 0	0 ± 0	0 ± 0	8.42 ± 0.43	48.01 ± 1.45	26.38 ± 0.86	23.9 ± 1.07	1.72 ± 1.02
	CP2%	0.29 ± 0.09	0.01 ± 0.01	0 ± 0	0 ± 0	7.53 ± 0.44	52.59 ± 2.75	25.04 ± 2.3	20.58 ± 0.91	1.79 ± 0.92
	P-value treatment	0.7929	0.3848	0.7354	ND	0.5288	0.3241	0.0927	0.2198	0.4079

ND, Not determined.

SCFAs = total amount of short-chain fatty acids (acetic + propionic + i-butyric + butyric + i-valeric + valeric acids; expressed as mg g^{-1} OM); acetic, propionic, and butyric acid proportions (expressed as % of total SCFAs).

BCFAs = branched-chain fatty acid proportions (i-butyric + i-valeric + valeric acids scaled to SCFAs, expressed as %).

Mean values (n = 8 animals) ± SEM.

[a,b]Mean values within a column with unlike superscript letters are significantly different ($p < 0.05$).

The P-values <0.05, $p < 0.01$, and $p < 0.001$ were considered as statistically significant, highly significant, and very highly significant.

not reveal any difference on d31–32 (**Supplementary Table 5**). *Enterococcus* spp. and unclassified *Christensenellaceae* were more abundant in the colon of piglets under IN supplementation compared to the other treatments. The colonic population of *Lachnospira* spp. was significantly higher with CP2% compared with IN and control diets. The genera *Clostridium* spp. and *Shuttleworthia* spp. were present in the lowest proportions with the CP2% treatment.

Experiment 2

In vivo Intestinal Permeability

At 7 days after dietary treatment, piglets showed lower permeability by decreased serum xylose concentrations when fed with IN (0.45 ± 0.03 mmol/L) in comparison to control (0.69 ± 0.11 mmol/L), CP0.2% (0.77 ± 0.10 mmol/L), and CP2% (0.83 ± 0.14 mmol/L) treatments ($p = 0.0868$).

DISCUSSION

In this study, we investigated the prebiotic potential of CP as a dietary strategy to alleviate the weaning stress of piglets in comparison to IN and its use as a well-recognized prebiotic. Consequently, the study design included a control group and a group supplemented with the minimum effective dose of IN, as a positive control for desired effects (34). For the experimental groups, supplementation with CP was done at the same dose as IN (0.2%) and at 2%, since the previously obtained results *in vitro* had shown that CP, a complex product, could not achieve the same effects at the same dose as IN, a pure compound (35, 42). So, it was decided to use a 10-fold higher dose of CP. Prebiotics aim to provide beneficial effects on animal health through the effects on the microbiota and derived metabolites in the digesta. Therefore, to investigate the effects of CP on gastrointestinal health, we determined an array of genes related to apoptosis, inflammation, and barrier integrity in the colon tissue, where an alteration in SCFA and BCFA was observed.

During the investigation period, IN supplementation did not induce an effect on the growth performance of the piglets, which contrasts with the results obtained from the studies on IN supplementation during the suckling period (43). Despite the absence of IN effects on performance, some differences were found in the health-related parameters measured. Morphological measurements of the intestinal epithelium showed that IN increased villus height to crypt depth ratios in the duodenum, jejunum, and ileum, together with numerically decreased crypt depths, which might be attributable to a reduced cell division in the crypts. This would be supported by the downregulated *CASP1* expression on d10–11, acting as a key enzyme in programmed cell death and cell turnover rate although no other upregulations or downregulations of apoptotic genes were found in this study. The reduced acidic goblet cell absolute counts in the small intestine of the IN treatments suggest a reduction in mucus production resulting in a thinner mucus layer and are in accordance with the lower gene expression level of *MUC2* in the colonic tissues of pigs fed with IN on d31–32. However, the possible effect on the mucus layer, which was also observed for the CP treatments, may suggest a higher susceptibility to bacterial translocation

TABLE 4 | Liquid chromatography-mass spectrometry (LC-MS) characterization of the main features using electrospray ionization (ESI) + and ESI – in the colonic content of piglets after feeding IN (IN0.2%) and CP (CP0.2% and CP2%) from post-weaning day 5 onward, at sampling times on d10–11 and d31–32 post-weaning. For the top compound ions, retention time (min) is shown, as well as the *p*-value and false discovery rate (FDR) for the differences between groups and the best identification hit.

Sampling time	ESI	Label	Retenion time	Mass-to-charge ratio	*p*-value	FDR	Best hit
d10–11	Pos	1	13.41	611.1972 m/z	3.15E-18	3.76E-15	Hesperidin
		2	19.51	457.2220 m /z	4.00E-17	2.55E-14	Unknown
		3	22.26	517.2432 m/z	1.61E-17	1.24E-14	Unknown
		4	24.21	439.2114 m/z	7.57E-19	1.75E-15	Glycocholic acid
		5	27.46	817.4587 m/z	6.72E-16	2.83E-13	Unknown
		6	12.34	273.0755 m/z	9.01E-15	2.32E-12	Naringenin
		7	24.66	403.1384 m/z	8.80E-18	8.16E-15	Unknown
		8	7.41	718.3002 m/z	1.78E-15	6.37E-13	Unknown
		9	8.03	615.2906 m/z	3.10E-14	6.84E-12	Unknown
		10	19.04	475.2323 m/z	6.90E-15	1.88E-12	Unknown
		11	19.39	473.2165 m/z	5.66E-15	1.64E-12	Unknown
		12	22.66	373.1279 m/z	9.89E-15	2.41E-12	Tangeritin
	Neg	1	13.32	609.1822 m/z	4.95E-14	1.34E-11	Hesperedin
		2	22.16	515.2283 m/z	5.55E-13	1.09E-10	Unknown
		3	16.86	593.1871 m/z	7.70E-17	5.55E-14	Isosakuranetin-7-O-rutinoside
		4	24.03	531.2233 m/z	4.24E-17	4.58E-14	Taurocholic/ursodeoxycholic acid
		5	7.34	716.2849 m/z	4.48E-15	1.61E-12	Frangulin A
		6	15.67	297.1330 m/z	7.23E-17	5.55E-14	Linarin
		7	19.39	646.1340 m/z	4.16E-14	1.20E-11	[DAla2] Leu-Enkephalin
		8	13.34	301.0699 m/z	3.69E-16	1.77E-13	Hesperidin
		9	12.09	915.2767 m/z	1.67E-12	3.00E-10	Neohesperidin
		10	9.12	787.2300 m/z	2.55E-16	1.39E-13	Naringenin-7-O-glucoside
		11	12.26	678.0876 m/z	1.17E-12	2.19E-10	Nariturin
		12	27.37	839.4668 m/z	1.75E-15	6.89E-13	Unknown
		13	13.59	359.0763 m/z	2.08E-12	3.59E-10	Gossypetin 3,3',8-trimethylether/4',5,7-Trihydroxy 3,6,8-trimethoxyflavone
		14	15.92	699.2222 m/z	7.96E-14	1.91E-11	Unknown
d31–32	pos	1	27.46	817.4587 m/z	7.38E-19	9.96E-16	Unknown
		2	19.51	457.2220 m/z	3.69E-18	2.14E-15	Unknown
		3	22.26	517.2432 m/z	1.26E-21	2.92E-18	Unknown
		4	24.21	439.2114 m/z	3.24E-18	2.14E-15	Glycocholic acid
		5	15.4	345.0967 m/z	2.73E-15	7.02E-13	Unknown
		6	18.78	735.3225 m/z	5.17E-16	2.00E-13	Unknown
		7	19.04	475.2323 m/z	2.18E-15	5.94E-13	Unknown
		8	24.66	403.1384 m/z	1.98E-16	9.16E-14	Unknown
		9	19.39	473.2165 m/z	1.60E-15	4.92E-13	Unknown
		10	16.03	701.2369 m/z	2.46E-16	1.03E-13	Unknown
		11	7.11	647.2267 m/z	1.31E-15	4.34E-13	Unknown
	neg	1	24.03	531.2233 m/z	1.31E-15	7.07E-13	Taurocholic/ursodeoxycholic acid
		2	13.32	609.1822 m/z	1.71E-14	4.93E-12	Hesperedin
		3	16.86	593.1871 m/z	1.60E-15	7.66E-13	Isosakuranetin-7-O-rutinoside
		4	13.34	301.0699 m/z	1.95E-16	1.41E-13	Hesperidin
		5	15.67	297.1330 m/z	1.93E-21	8.34E-18	Linarin
		6	12.26	678.0876 m/z	8.28E-14	1.79E-11	Nariturin
		7	9.12	787.2300 m/z	1.60E-14	4.93E-12	Naringenin-7-O-glucoside
		8	20.93	517.4578 m/z	1.09E-15	6.72E-13	Unknown
		9	27.37	839.4668 m/z	2.28E-15	9.86E-13	Unknown
		10	18.66	711.3234 m/z	1.71E-16	1.41E-13	18-Hydroxy-5Z,8Z,11Z,14Z,16E-eicosapentaenoic acid
		11	19.39	646.1340 m/z	8.75E-15	2.91E-12	[DAla2] Leu-Enkephalin
		12	15.92	699.2222 m/z	5.94E-14	1.36E-11	Unknown
		13	12.09	915.2767 m/z	1.53E-16	1.41E-13	Neohesperidin
		14	9.82	718.3000 m/z	3.68E-14	9.94E-12	Unknown

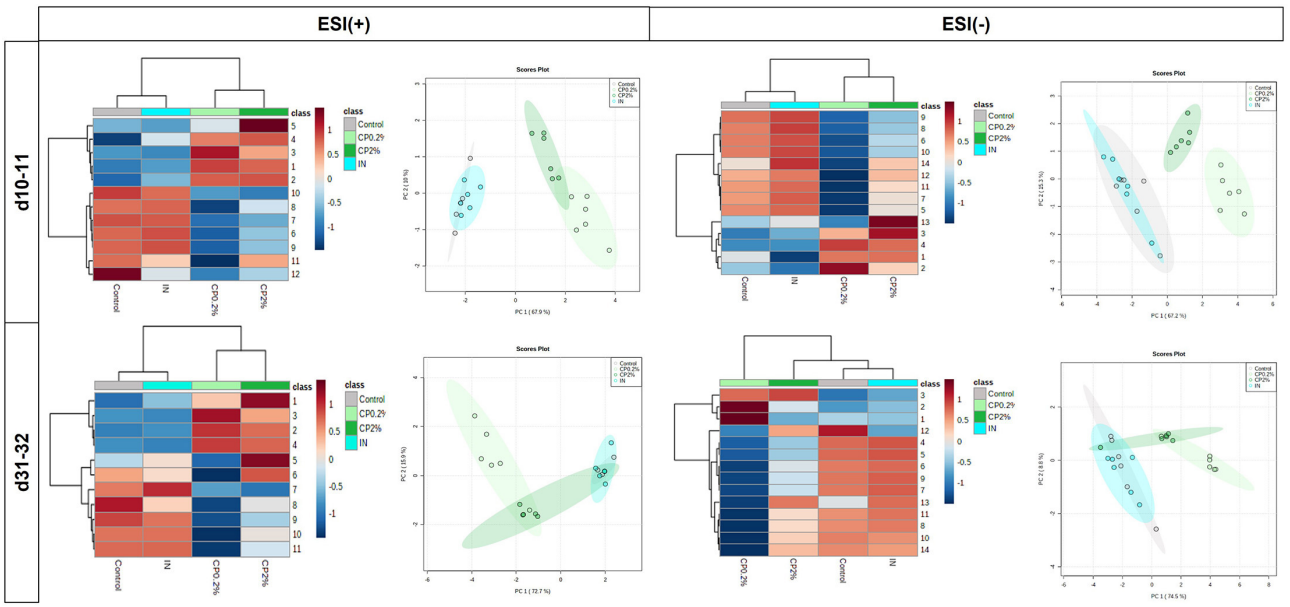

FIGURE 5 | Heatmap (left) and PCA plots (right) of metabolomic analysis in the colon after feeding a diet supplemented with IN (IN0.2%) and CP (CP0.2% and CP2%) from post-weaning day 5 onward, at sampling times on d10–11 (top) and d31–32 (bottom) post-weaning. The results show the top most significant compound ions ($p < 0.05$) obtained from an analysis by liquid chromatography-mass spectrometry (LC-MS) (positive "+" and negative "–"; top left and right, respectively). Numbers refer to the labels used in **Table 4** presenting the different compounds. The groups compared were control, IN, and CP (CP0.2% and CP2%). Each colored cell on the heatmap corresponds to the average concentration of the compound (row) per treatment group (column; $n = 6$ per group).

TABLE 5 | Gas chromatography-mass spectrometry (GC-MS) characterization of the main features in the colonic content of piglets after feeding IN (IN0.2%) and CP (CP0.2% and CP2%) from post-weaning day 5, at sampling times on d10–11 and d31–32 post-weaning.

Sampling time	Label	Compound	*p*-value	FDR	Library (NIST) Match	Score
d10–11	1	Compound262_33.36	0.010469	0.98931	Unknown	–
	2	Compound260_33.25	0.015944	0.98931	13-Trimethylsilyloxy-9-octadecenoic acid, methyl ester	77.09
	3	Compound235_30.07	0.010681	0.98931	13-Octadecenoic acid, methyl ester	82.93
	4	Compound60_14.84	0.045133	0.98931	Unknown	–
	5	Compound32_12.3	0.017057	0.98931	Unknown	–
	6	Compound79_17.35	0.049825	0.98931	Unknown	–
	7	Compound232_29.86	0.040642	0.98931	Unknown	–
	8	Compound55_14.62	0.01473	0.98931	Unknown	–
	9	Compound205_27.21	0.031758	0.98931	3-indoleacetic acid, 2TMS derivative	80.88
d31–32	1	Compound23_11.34	0.011181	0.69467	Unknown	–
	2	Compound235_30.07	0.044674	0.96277	13-Octadecenoic acid, methyl ester	83.2
	3	Compound190_26.05	0.0054548	0.59639	Unknown	–
	4	Compound303_41.41	0.00037716	0.12371	Unknown	–
	5	Compound57_14.66	0.0046701	0.59639	Unknown	–
	6	Compound153_23.34	0.012707	0.69467	Methyl-galactoside (1S, 2S, 3S, 4R, 5R)-, 4TMS derivative	83.41
	7	Compound296_39.78	0.031652	0.96277	Lactose, 8TMS derivative	83.61
	8	Compound50_13.85	0.010807	0.69467	Pipecolic acid, 2TMS derivative	75.73
	9	Compound327_49.72	0.034089	0.96277	Unknown	–

For the top compound ions, retention time (min) is shown, as well as the p-value and FDR for the differences between groups and the best identification hit.

(44). Furthermore, both CP treatments reduced the acidic goblet cell counts in the small intestine indicating a lower mucus production, in line with Hedemann et al. (45) and Swiech et al. (46). Interestingly, no modification of the mucin 1 gene (*MUC1*) was observed in this study, indicating the difficulty of data interpretation as not all parameters always go in the same line. However, in the theoretical event of bacterial translocation, a subsequent inflammatory response would occur, which was not

TABLE 6 | Microbial alpha diversity after feeding IN (IN0.2%) and CP (CP0.2% and CP2%) from post-weaning day 5 onward, at sampling times on d10–11 and d31–32 post-weaning.

Indexes	Sampling time	Control	IN0.2%	CP0.2%	CP2%	*P*-value treatment
Chao1	d10–11	1,069.52 ± 115.20	1,241.23 ± 84.18	1,122.04 ± 92.40	1,204.28 ± 156.77	NS
	d31–32	1,202.98 ± 121.84	1,267.39 ± 135.22	1,207.93 ± 114.67	1,179.61 ± 79.73	NS
Observed						
OTU	d10–11	617.56^{b} ± 92.31	754.14^{a} ± 45.35	659.58^{b} ± 50.62	708.55^{ab} ± 85.25	**<0.05**
	d31–32	722.10 ± 80.76	762.98 ± 98.10	733.11 ± 63.25	712.33 ± 52.63	NS
PD whole tree	d10–11	40.13^{ab} ± 5.28	47.00^{a} ± 2.82	41.34^{b} ± 4.00	43.62^{ab} ± 5.67	**<0.05**
	d31–32	44.99 ± 4.95	47.97 ± 5.97	46.56 ± 3.93	43.75 ± 3.03	NS
Shannon	d10–11	5.92 ± 0.44	6.40 ± 0.22	6.21 ± 0.22	6.39 ± 0.37	NS
	d31–32	6.49 ± 0.22	6.57 ± 0.39	6.42 ± 0.47	6.42 ± 0.27	NS

OTU, operational taxonomic unit; PD phylogenetic diversity.
Mean values (n = 8 animals) ± SD.
[a,b]Mean values within a row with unlike superscript letters are significantly different ($p < 0.05$).
The P-values <0.05, $p < 0.01$, and $p < 0.001$ were considered as statistically significant, highly significant, and very highly significant.

observed in our study, seen the lack of differential colonic gene expression of the inflammatory genes. Furthermore, intestinal permeability *in vivo*, used as a marker for intestinal integrity, showed a tendency to decrease, implying increased integrity of the intestinal barrier, due to the IN treatment after 1 week and controversly lower levels of occludin on d31–32 *in vivo*. This would be in line with previous research showing that *in vitro* batch fermentation of IN using feces of the piglet produced increased levels of SCFA and butyrate, together with an upregulation of tight and adherens junction genes in IPEC-J2 cells (47). In general, IN fermentation is considered to occur mainly within the distal end of the small intestine in pigs (48), which may indicate a reduced capacity of the ingredient to modify porcine hindgut fermentation (34), and therefore, corresponds with our lack of response in the colonic and cecal metabolites and minor microbiota modulation following IN supplementation. Therefore, as several parameters pointed to a positive effect of IN, our data are in line with the general positive known effects of IN on gut health in livestock, such as increased weight and VH:CD ratio, reduced inflammation, improved gut barrier function, and a decrease of pathogenic bacteria abundance (30, 32, 33, 43). Moreover, IN supplementation has been shown to modulate immune responses, improve the gut barrier function, and favor antidiabetogenic microbiota in mice (49). Still, future investigations should also consider these targets on permeability and inflammation at the protein level for a better understanding of the underlying mechanisms.

The absorptive capacity and function of the intestine were shown to be affected by both doses of CP, as was the case for IN, with increased villus height to crypt depth ratios in the duodenum, jejunum, and ileum; which was related to decreased crypt depths. Similarly, a reduced mitotic count was observed in crypts after dietary pectin supplementation (41), and it has also been related to higher luminal viscosity (16), which was observed in the ileum of the CP2% treatment on d31–32. Conflicting results about the impact of pectin-rich ingredients on the small intestinal morphology have been documented in the weaned pigs (22, 41), but they could be explained by a dose-effect. At the animal level, the effect of CP was translated into temporary softer feces in week 2 for the CP2% treatment, and a higher FCR in the 1st week, without remaining or global effects for the entire investigation period. This higher FCR could also be explained by a higher villi height to crypt depth ratio, implying an increased absorptive surface with reduced proliferative activity.

Intestinal permeability was shown to remain unaltered when comparing between treatments, even though CP at 0.2% decreased mRNA levels of intestinal barrier integrity-related genes such as *claudin-3* and *MUC2* on d31–32 post-weaning in colonic tissue. The investigation of the inflammatory responses in the duodenum and jejunum might provide novel information and highlight correlations between these parameters.

At d10–11 post-weaning, after 5–6 days of CP supplementation, the 11 inflammation signaling target genes related to the AKT, MAPK, and TLR pathways remained unaffected by the different diets. The other 14 inflammatory target genes, including cytokines, chemokines, and defensins, did not differ between the control and the supplemented diets in colonic tissues at the first sampling time. The gene expression of *DEFβ4a*, encoding for the β-defensin 2, was downregulated in colonic tissues following the IN and CP2% inclusion on d31–32 of the treatment supplementation, supposing a modest impact of these two treatments on the intestinal pro-inflammatory status. Other immunity-related genes, including the inflammation signaling pathway target genes but also cytokines and chemokines, remained unaffected by the treatments at the second sampling time, which is in line with Weber et al. (26) when supplementing the weaned piglets for a week with 7.5% CP.

Concerning the fermentation of the CP, we determined the targeted fermentation metabolites, namely SCFA, intermediary compounds, and BCFA, resulting from the fermentation of the bacteria residing in the ileum, cecum, and colon. None of these fermentation metabolites were affected by the dietary treatments, for both sampling times in the ileum nor the cecum, suggesting

TABLE 7 | Microbiota composition in the colon after feeding a diet supplemented IN (IN0.2%) and CP (CP0.2% and CP2%) from post-weaning day 5 onward, at sampling times on d10–11 and d31–32 post-weaning.

Sampling time	Phylum / Genus	*P*-value	FDR	Control	IN0.2%	CP0.2%	CP2%	SEM
d10–11	***Bacteroidetes***	NS	NS	26.9	25.2	26.6	25.7	1.4
	Unclassified *Paraprevotellaceae*	NS	NS	0.7	1.3	1.5	1.9	0.3
	Prevotella	NS	NS	23.7	21	22.9	21.7	1.7
	S24-7	NS	NS	1.5	1.3	1	1.1	0.5
	Firmicutes	NS	NS	70.3	71.9	70.3	72	1.7
	Lactobacillus	NS	NS	26.5	23.3	25.1	21.1	3.7
	Streptococcus	NS	NS	1.2	0.9	0.8	0.9	0.5
	Unclassified *Clostridiales*	NS	NS	2	3.3	3	2.7	0.3
	Unclassified *Clostridiaceae*	NS	NS	1.2	4.4	1.8	3.5	1
	SMB53	NS	NS	0.3	1.3	0.3	0.6	0.3
	Unclassified *Lachnospiraceae*	NS	NS	3.6	3.9	3.9	3.7	0.3
	Blautia	NS	NS	4.4	3.5	3.1	4.6	0.6
	Coprococcus	NS	NS	0.8	1.3	1	1.2	0.2
	Roseburia	NS	NS	4.5	6.9	6.1	6.4	1
	Lachnospiraceae Other	NS	NS	0.8	1.2	1	1.3	0.2
	Unclassified *Ruminococcaceae*	NS	NS	6.9	7.2	6.5	8.9	0.8
	Faecalibacterium	**0.04**	NS	2.4 ^{b}	2.7 ^{b}	2.4 ^{b}	3.6 ^{a}	0.4
	Ruminococcus OTU2	NS	NS	1.5	1.8	1.5	1.5	0.3
	Anaerovibrio	**0.04**	NS	1.3 ^{b}	2.2 ^{a}	3.2 ^{a}	1.5 ^{ab}	0.5
	Megasphaera	NS	NS	3.9	1.3	1.9	2	0.6
	Phascolarctobacterium	NS	NS	1.1	0.9	1.1	1.4	0.2
	Eubacterium	NS	NS	1	0.6	0.7	1	0.2
	Catenibacterium	NS	NS	1.1	0.2	1	0.7	0.5
	Proteobacteria	NS	NS	2	1.4	2.2	1.1	0.5
	Succinivibrio	NS	NS	1.1	0.3	1.1	0.2	0.4
d31–32	***Bacteroidetes***	NS	NS	27.5	25.9	27.2	27.5	1.2
	Unclassified *Bacteroidales*	NS	NS	0.8	1.2	1.2	0.8	0.2
	Unclassified *Paraprevotellaceae*	NS	NS	1.7	1.5	1.2	1.5	0.3
	Prevotella	NS	NS	23.4	21.1	23.3	23.5	1.3
	S24-7	NS	NS	1.1	1.4	1.1	1.4	0.3
	Cyanobacteria	**0.00**	**0.01**	1.3 ^{a}	0.5 ^{b}	0.9 ^{ab}	0.3 ^{b}	0.2
	Unclassified *2YS2*	**0.00**	**0.04**	1.3 ^{a}	0.5 ^{b}	0.9 ^{ab}	0.3 ^{b}	0.2
	Firmicutes	NS	NS	68.7	71.8	70	70.7	1.5
	Lactobacillus	NS	NS	11.6	15.8	13.1	17.4	4.1
	Streptococcus	NS	NS	2	1.4	2.3	3.7	0.9
	Unclassified *Clostridiales*	NS	NS	3.6	2.8	3.8	2.8	0.4
	Unclassified *Clostridiaceae*	NS	NS	7.6	7.1	7.9	4.4	1.7
	SMB53	NS	NS	1.2	1	1.4	0.7	0.3
	Unclassified *Lachnospiraceae*	**0.02**	NS	4.9 ^{a}	4.6 ^{a}	3.8 ^{ab}	3.4 ^{b}	0.4
	Blautia	NS	NS	3.4	3.7	2.6	3.3	0.4
	Coprococcus	NS	NS	1.3	1.3	1.2	1.1	0.1
	Dorea	NS	NS	0.9	1.1	0.7	1	0.2
	Roseburia	NS	NS	6.9	5.3	7.5	7	1
	Lachnospiraceae Other	NS	NS	1.6	1.4	1.3	1.6	0.2
	Unclassified *Ruminococcaceae*	NS	NS	7.5	10.2	8.2	8.5	0.7
	Faecalibacterium	**0.03**	NS	1.8 ^{b}	2.4 ^{ab}	2.2 ^{b}	3.2 ^{a}	0.4
	Ruminococcus OTU2	NS	NS	1.9	1.6	1.7	1.6	0.2
	Anaerovibrio	NS	NS	2	1.5	1.8	1.4	0.4
	Megasphaera	**0.03**	NS	1.0 ^{b}	1.4 ^{b}	1.4 ^{b}	2.2 ^{a}	0.3
	Phascolarctobacterium	NS	NS	1.1	1.4	1.3	1.1	0.1
	Eubacterium	NS	NS	1.4	0.8	0.9	0.8	0.4
	Bulleidia	**0.01**	NS	0.5 ^{b}	1.3 ^{a}	0.9 ^{a}	0.3 ^{b}	0.3
	Proteobacteria	NS	NS	1	0.6	0.7	0.6	0.3

OTU, operational taxonomic unit.

The microbiota composition is expressed as a percentage (%) of the total microbiota.

Only genera and phyla with a relative abundance ≥1% for one or more treatment(s) were included in this table.

Mean values (n = 8 animals).

^{a,b}Mean values within a row with unlike superscript letters are significantly different ($p < 0.05$).

that the complex carbohydrate fraction of CP may shift the fermentation site to more distal regions of the digestive tract (17). Indeed, in the colonic digesta on d10–11, a significant increase in acetate proportions resulting from CP2% inclusion was observed, which is in concordance with the results of Moset et al. (50) and Pascoal et al. (51). Other studies reported acetate as the major end product of pectin fermentation (52), which is in line with our results. Concomitant with the rise in acetate proportions, BCFA levels were decreased in the colon of piglets fed with CP2% on d10–11, which is in agreement with the lower valerate concentrations found by Moset et al. (50) and Almeida et al. (25) in the weaned piglets. This result may suggest that the inclusion of CP in the diet of newly weaned piglets as an ingredient could reduce proteolytic fermentation in the hindgut shortly after weaning. The inclusion of CP0.2% did not cause any significant change in SCFA proportions, thereby the modulation of metabolites by CP was assumed to be dose-dependent.

For the determination of metabolites using LC-MS, a clear separation can be seen mainly between the CP groups and the other two groups, i.e., the control group and the IN group. At both sampling times, hesperidin and neohesperidin were identified by LC-MS as significantly more present in both CP treatments, which is not surprising as these flavonoids are the main compounds in citrus fruits (53). In a study where neohesperidin was given to piglets around the time of weaning, an enhanced expression of Na+/glucose cotransporter SGLT1 was observed, increasing the intestinal capacity to absorb glucose and avoiding nutrient malabsorption (54), and an increase in the cecal population abundance of *Lactobacillus* spp. (55). However, in our study, the supplementation of CP did not significantly alter the colonic abundance of *Lactobacillus* spp. although we did observe changes in colonic gut microbiota, which is consistent with the hypothesis that CP is mainly fermented in the hindgut (24). Nevertheless, the statistical significances disappeared after the FDR correction. Indeed, pigs offered CP2% showed higher colonic levels of *Faecalibacterium* spp. at both sampling times, *Megasphaera* spp. only at d10–11 sampling time and *Anaeribio spp.* only at d31–32 sampling in comparison to control counterparts, which are in line with those obtained by Bang et al. (56) in a study using pectin in human donors. Indeed, taxa of the *Lachnospiraceae* family (57) and the *Faecalibacterium* spp. genus (58, 59) were credited with pectin-degrading capacities, resulting in galacturonic acid and acetate productions (56). We suggest that the rise in acetate-producing bacterial populations like *Lachnospira* spp. may have led to an enhancement of other non-pectic saccharolytic bacteria, such as *Megasphaera* spp. and *Ruminococcus* spp., *via* cross-feeding processes. It should be emphasized that the CP0.2% treatment showed the lowest abundances of *Faecalibacterium* spp., *Megasphaera* spp., and *Ruminococcus* spp., suggesting, once again, a dose-dependent effect of CP.

The metabolites determined by GC-MS showed differences when looking at the top compounds between the control group and the treatment groups, and some interesting compounds were identified although only significant by *P*-value and becoming insignificant after the FDR correction. Octadecenoic acid was found in higher amounts in all treatment groups at d10–11, which is a compound generated by lactic acid bacteria in the gut and has been described to provide protection against oxidative stress cytotoxicity (60). At d10–11 sampling time, the compound 3-IAA was detected in all samples from CP-treatment groups and only in two to three samples in control and IN groups, which is a microbiota-derived tryptophan catabolite produced in the colon by bacteria such as *Lactobacillus* (61, 62), and it has been shown to reduce susceptibility to lipopolysaccharide (LPS) (61), to promote anti-inflammatory responses (63), and to be involved in the maintenance of intestinal immune cells *via* AhR signaling (62). Finally, pipecolic acid was identified at d31–32 sampling time in all individuals at higher amounts in control and CP0.2% treatment groups. Pipecolic acid is a bacterial-derived lysine catabolite that has been related to increased feed efficiency in pigs (64), which, from our results, could indicate a decreased proteolytic fermentation due to IN and CP2% treatments. Despite the absence of statistically significant differences between groups, the identified compounds proved to be of high interest, and further research would be necessary to draw conclusions.

In conclusion, CP modulated the fermentation processes in the hindgut, as seen by an enhancement of several health-related bacterial populations for both periods in the colon of the CP2% treatment, together with higher acetate and lower BCFA proportions. The altered small intestinal morphology and acidic goblet cell count for both IN- and CP-supplemented pigs demonstrate effects on the host that merit further investigation. Nevertheless, a challenging trial with *E. coli* could provide new insights into the preventive or curative properties of CP against pathogen infection, under the conditions found at weaning in commercial pig productions.

DATA AVAILABILITY STATEMENT

Raw sequences have been uploaded in the European Nucleotide Archive database (project number PRJEB38284).

ETHICS STATEMENT

These animal experiments were approved by Animal Ethical Committee of the University of Liège and the University of Ghent.

AUTHOR CONTRIBUTIONS

JU, NE, JB, MS, GB, JW, and GP: conceptualization, methodology, supervision, project administration, and validation. JU, EA, KK, MS, ST, and MD: research conduction. NE and GB: funding acquisition and resources. JU, EA, and MS: investigation and writing—original draft. All authors: review and editing.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Modulation of Gut Microbiota and Immune System by Probiotics, Pre-biotics, and Post-biotics

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The human gastrointestinal tract harbours a complex microbial community, which interacts with the mucosal immune system closely. Gut microbiota plays a significant role in maintaining host health, which could supply various nutrients, regulate energy balance, modulate the immune response, and defence against pathogens. Therefore, maintaining a favourable equilibrium of gut microbiota through modulating bacteria composition, diversity, and their activity is beneficial to host health. Several studies have shown that probiotics and pre-biotics could directly and indirectly regulate microbiota and immune response. In addition, post-biotics, such as the bioactive metabolites, produced by gut microbiota, and/or cell-wall components released by probiotics, also have been shown to inhibit pathogen growth, maintain microbiota balance, and regulate an immune response. This review summarises the studies concerning the impact of probiotics, pre-biotics, and post-biotics on gut microbiota and immune systems and also describes the underlying mechanisms of beneficial effects of these substances. Finally, the future and challenges of probiotics, pre-biotics, and post-biotics are proposed.

Keywords: probiotics, pre-biotics, post-biotics, gut microbiota, immune system

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INTRODUCTION

Over 1,000 bacterial species exist within the human gut, with more than 50 bacterial genera being described (1). The large intestine harbours the highest numbers of bacteria in the gastrointestinal tract, at around 10^{11}-10^{12} cells per gramme (2). Gut microbiota plays a significant role in maintaining host health, which could supply various nutrients, regulate energy balance, modulate the immune response, and defence against pathogens (3). Therefore, maintaining a favourable equilibrium of gut microbiota is beneficial to host health. Several studies have found that pre-biotics, probiotics, and probiotic-driven metabolites, known as "post-biotic," are able to improve intestinal microbiota homeostasis, maintain gut barrier integrity, and modulate immune response (**Figure 1**), and exert beneficial effect to host health by preventing against pathogen invasion and risks of obesity, type 2 diabetes, inflammatory bowel disease, cancer, cardiovascular, liver, and central nervous system disorders (4).

EFFECTS OF PROBIOTICS ON GUT MICROBIOTA AND IMMUNE FUNCTION

At the beginning of the 1900s, Metchnikoff firstly introduced the scientific probiotic concept and hypothesised that longevity in Bulgarian peasants stemmed from their large intakes of fermented milk containing health-promoting microorganisms (now known as probiotics) (5). In 2001 and 2002, probiotics were defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" by a WHO/FAO working party (6). In 2014, probiotics were stipulated as "defined contents, appropriate viable count at end of shelf life, and suitable evidence for health benefits" and further stated that all probiotics must be "safe for their intended use" by Hill et al. (6). These criteria were then reiterated by the International Scientific Association of Probiotics and Pre-biotics (ISAPP) in a position statement in 2018 (5). Up to now, the list of probiotics that can be used in food includes 35 species or subspecies and has been divided into three categories: lactic acid-producing bacteria (*Lactobacillus, Bifidobacterium,* and *Enterococcus*), yeast, and spore-forming *Bacillus* species. *Bifidobacterium* spp. and *Lactobacillus* spp. are generally isolated from healthy human colon or dairy sources (7).

Effects of Probiotics on Gut Microbiota

It is necessary to maintain the balance of the gastrointestinal microbiome, which plays a significant role in many diseases. The gastrointestinal epithelium is the first point of host contact with microorganisms, the infection, and invasion of any pathogenic bacteria will contact the mucosa of the intestinal tract and activate immune response (8). Changes in the composition and diversity of microflora are associated with several gastrointestinal diseases

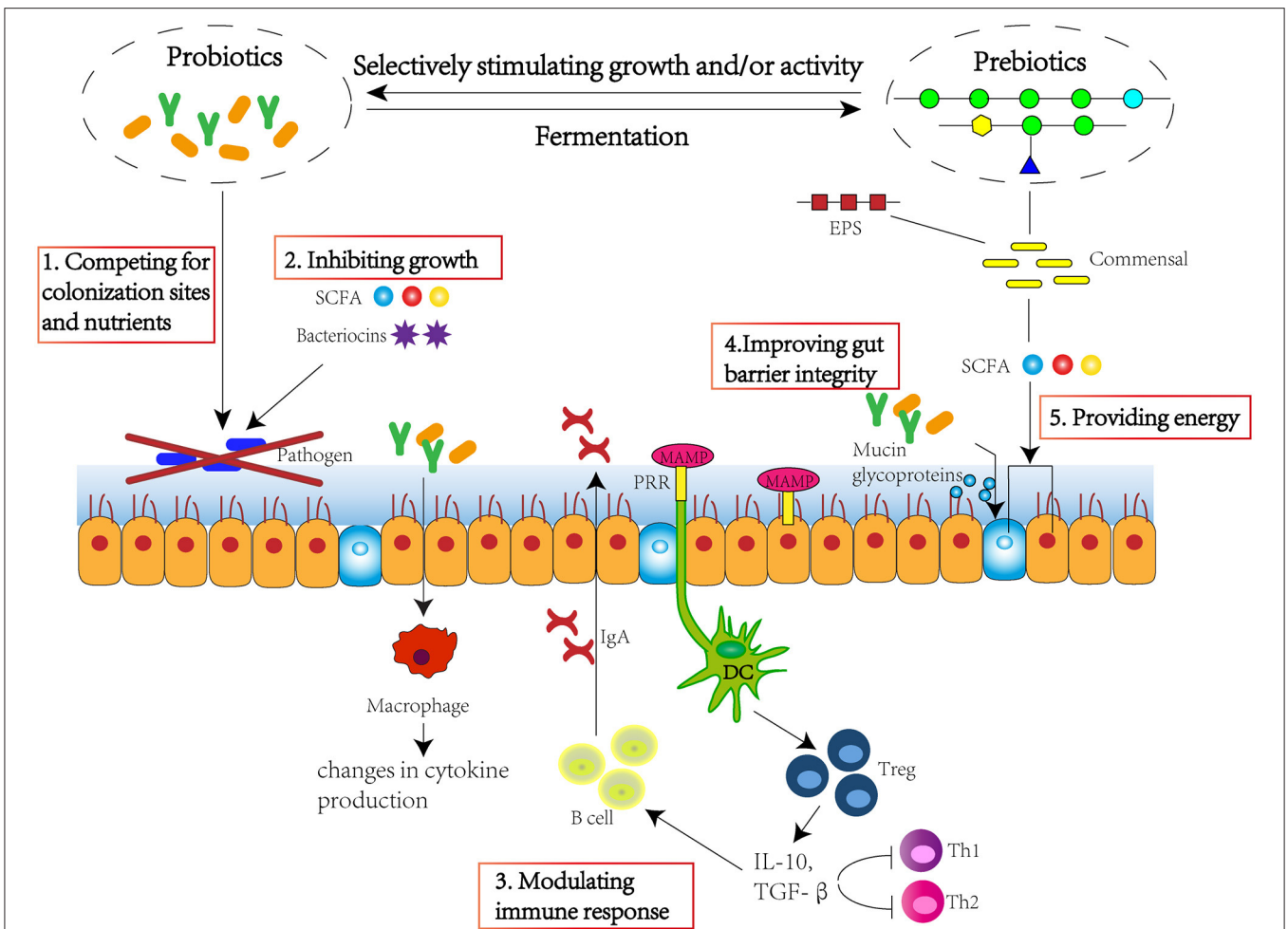

FIGURE 1 | Pre-biotics, probiotics, and post-biotics are able to improve intestinal microbiota homeostasis, maintain gut barrier integrity, and modulate immune response. The approach mechanisms include 1. competing for colonisation sites and nutrients; 2. inhibiting growth through production of SCFA and bacteriocins; 3. modulating immune response through stimulating immune cells and cytokine and immunoglobulin production; 4. improving gut barrier integrity through stimulating the production of mucin glycoproteins; 5. providing energy for epithelium, improving villi growth, crypt development, tight junctions, and mucin production. SCFA, short-chain fatty acid; EPS, exopolysaccharide; MAMP, microorganism-associated molecular patterns; PRR, pattern recognition receptor; DC, dendritic cell; Treg, regulatory T cell; IL-10, interleukin 10; TGF-β, transforming growth factor β.

(inflammatory bowel disease, colorectal cancer, or irritable bowel syndrome) and parenteral diseases, such as allergies, bronchial asthma, and cystic fibrosis (9, 10).

Probiotics have been shown to influence gut microbiota in animal studies and human clinical trials (**Table 1**). *Bifidobacterium* spp. are primary beneficial bacteria inhabiting in human intestines, and the alterations in the number and composition of microbiota are one of the most common features of diseases, such as Crohn's disease (24), ulcerative colitis (25), respiratory infection disease (11), and autism (26). Probiotics could modulate human intestinal bacteria, inhibiting harmful bacteria, such as *Desulfovibrio*, and stimulating beneficial bacteria, such as lactic acid bacteria. In one study, probiotic Bifico (*Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Enterococcus faecalis*, and *Bacillus cereus*) pre-treatment has been found to reduce the risks of colitis-associated cancer and formation of tumour in mice, through decreasing the abundance of colitogenic bacteria, such as *Desulfovibrio*, *Mucispirillum*, and *Odoribacter*, and increasing the abundance of *Lactobacillus* (12). In another animal study, probiotic yeast *Saccharomyces boulardii* (*S. boulardii*) was found to significantly increase the abundance of *Bacteroidetes* and decrease the abundance of *Firmicutes*, *Proteobacteria*, and *Tenericutes*, which were correlated with the host metabolism alterations, and indicated the potential role of *S. boulardii* in the treatment of obesity and type 2 diabetes (13). The impacts of probiotics on gut microbiota are also investigated and proved in human clinical trials. In a randomised, double-blind, and placebo-controlled human dietary intervention study, three different doses [5×10^9 CFU/day (high), 1.0×10^9 CFU/day (medium), and 6.5×10^7 CFU/day (low)] of *B. lactis* HN019 were found to significantly increase levels of bifidobacteria and lactobacilli in elderly compared to placebo. Importantly, this study demonstrated that the lowest dose of *B. lactis* HN019 (6.5×10^7 CFU/day) was effective in microbiota modulation in the elderly, provided *B. lactis* HN019 consumption guidelines (14). The mixture of probiotic strains not only showed gut microbiota modulatory effect in healthy volunteers (15) but also in volunteers with recurrent respiratory tract infection disease (11). According to these studies, probiotics could regulate and restore gut microbiota balance through stimulating the growth/activity of beneficial bacteria and suppressing those of harmful bacteria. Although the underlying mechanism of probiotics on gut microbiota modulation remains unclear, probiotics could inhibit the growth of pathogens through the production of short-chain fatty acids (SCFA) and toxins (27) and competition of colonisation sites with pathogens (28). In addition, as more next-generation probiotic strains are identified, the strain-specific effect and effective dose of them need to be investigated.

Effects of Probiotics on Immune Function

Pattern recognition receptors, such as toll-like receptors (TLR), which are expressed on immune cells, could be recognised by probiotics, after which probiotics could regulate important signalling pathways, producing nuclear factor κb (NF κb) and mitogen-activated protein kinases, and communicate with the host (29). In addition, the innate immune response could be activated, resulting in the production of pro- and anti-inflammatory cytokines or chemokines (8). The effects of probiotics on immunity are summarised in **Table 1**.

In an *in vitro* study, four different strains of probiotics [*Lactobacillus rhamnosus* (*L. rhamnosus*) GG, *L. rhamnosus* KLSD, *Lactobacillus helveticus* (*L. helveticus*) IMAU70129, and *Lacticaseibacillus casei* (*L. casei*) IMAU60214] could stimulate innate immunity by increasing phagocytosis of human monocyte-derived macrophages, levels of reactive oxygen species (ROS), and signalling of NF-κB pp65 and TLR2 (16). Another *in vivo* study also found similar results, the pre-treatment of *Lactobacillus johnsonii* (*L. johnsonii)* NBRC 13952 could enhance the phagocytosis of macrophage cell line RAW264.7 on various pathogens and promote the expression of interleukin-1β (IL-1β) and CD80 (30). The regulatory effects were further investigated in animal and human studies. In an animal study, mice orally administrated with *Lactobacillus gasseri* (*L. gasseri*) SBT2055 (LG2055) were found to have increased production of IgA and numbers of IgA+ cells in Peyer's patches and lamina propria, which might result from the stimulation of transforming growth factor β (TGF-β) expression and activation of TLR2 signalling pathways (17). Kwon et al. (18) applied the animal model and found probiotics mixtures *L. acidophilus, L. casei, Limosilactobacillus reuteri* (*L. reuteri*), *Bifidobacterium bifidum* (*B. bifidium*), *and Streptococcus thermophilus* (5×10^8 cfu/day) could increase numbers of CD4 + Foxp3+ regulatory T cells (Tregs) and decrease numbers of T helper (Th) 1, Th2, and Th17 cytokines, contributing to inhibit progression and immune disorders in inflammatory bowel disease, atopic dermatitis, and rheumatoid arthritis. *Bifidobacterium breve* (*B. breve*) AH1205 and *Bifidobacterium longum* (*B. longum*) AH1206 also could promote the expression of transcription factor Foxp3 to induce Tregs in infant mice, which were associated with a protective effect against allergy (31). A human clinical trial also showed that healthy infants fed with infant formulas with *B. infantis* R0033, *Bifidobacterium bifidum* (*B. bifidum*) R0071, and *L. helveticus* R005 would have a higher level of faecal secretory IgA (sIgA) compared to control, which were associated with enhanced mucosal immunity (9). In addition, probiotic *B. infantis* 35624 has been shown to increase the proportion of $Foxp3^+$ lymphocytes in peripheral blood in healthy volunteers and decrease levels of pro-inflammatory cytokines and C-reactive protein in psoriasis patients, chronic fatigue syndrome patients, or ulcerative colitis patients (32, 33). Childs and Röytiö (19) conducted a double-blind, placebo-controlled, randomised, factorial cross-over study to investigate the impact of probiotic *Bifidobacterium animalis* (*B. animalis*) on immune response and found that probiotics combined with xylo-oligosaccharide could reduce the expression of CD19 on B cells. These findings suggested the crucial role of probiotics in immune function regulation, not only in healthy but also in disease patients through activating important immune signalling pathways and modulating the activity of immune cells. Importantly, the role of probiotics as alternative supplementation in disease treatment and the underlying mechanisms need to be further investigated.

TABLE 1 | Example of studies designed to determine effects of probiotics on gut microbiota and immune function.

Subjects	Substrates	Dose	Duration	Results	References
66 healthy infants	Infant formulas (*Bifidobacterium infantis* R0033, *B. bifidum* R0071, and *Lactobacillus helveticus* R0052)	Feeding >80% of daily food	4 weeks	High levels of faecal sIgA, suggesting a positive effect of probiotics on sIgA production	(9)
120 children	*Bifidobaeterium* tetravaccine tablets (*B. infantis, L. acidophilus, Enterococcus faecalis* and *Bacillus cereus*)	3 tablets/12 h	2 months	The number of *Bifidobacterium* and *Lactobacillus* was significantly higher	(11)
35 C57BL/6 mice	Probiotic cocktail Bifico (*B. longum*, *L. acidophilus*, *Enterococcus faecalis*)	1.2×10^7 CFU/d	9 weeks	Bifico decreased the abundance of genera *Desulfovibrio, Mucispirillum,* and *Odoribacter*, and a bloom of genus *Lactobacillus* was detected	(12)
30 6-week-old db/db mice	*Saccharomyces boulardii Biocodex*	120 mg/d	4 weeks	Significantly change the gut microbiota composition with an increased abundance of *Bacteroidetes* and a decreased abundance of the phyla *Firmicutes*, *Proteobacteria*, and *Tenericutes*	(13)
80 elderly people	*B. lactis* HN019	5×10^9 CFU/d, 1.0×10^9 CFU/d, and 6.5×10^7 CFU/d	4 weeks	All the three doses caused a significant increase in *Bifidobacteria, lactobacilli* and *enterococci* and a decrease in *Enterobacteriaceae*	(14)
20 healthy Italian volunteers	*B. longum* BB536, *L. rhamnosus* HN001	4×10^9 CFU/d	4 weeks	A higher abundance of *Blautia producta, Blautia wexlerae* and *Haemophilus ducrey* was observed, together with a reduction of *Holdemania filiformis, Escherichia vulneris, Gemmiger formicilis* and *Streptococcus sinensis abundance*	(15)
Macrophages derived from monocytes	*L. rhamnosus* GG, *L. rhamnosus* KLSD, *L. helveticus* IMAU70129, *and L. casei* IMAU60214	10^8 CFU/mL/d	24 h	Improve the phagocytosis and bactericidal activity such as *S. aureus, S. typhimurium,* and *E. coli* (*Staphylococcus aureus*, *Salmonella typhi-murium*, *E. coli*)	(16)
20 Balb/c mice	*L. gasseri* SBT2055 (LG2055)	1.0×10^9 CFU/g	5 weeks	An increased production of IgA and numbers of IgA+ cells in Peyer's patches and lamina propria	(17)
30 BALB/c mice	*L. acidophilus, L. casei, L. reuteri, B. bifidium, and Streptococcus thermophilus*	5×10^8 CFU/d	20 days	Increased numbers of $CD4^+Foxp3^+$ regulatory T cells, and decrease numbers of Th 1, Th2, and Th17 cytokines	(18)
44 healthy adults	*B. animalis*	10^9CFU/d	21 days	Probiotic combined with xylo-oligosaccharide could reduce the expression of CD19	(19)
47 healthy women	Total bacteria in human milk	1.5 to 4.0 log^{10}CFU/mL	24 h	No potential probiotics were found to antagonise pathogens, but they all agglutinate different pathogens	(20)
20 BALB/c mices	*Lactobacilli* and *Bifidobacteria*	5×10^9 CFU/mL/d	6 days	Blocking autophagy *in vitro* reduces the IL-10 and exacerbates the secretion of IL-1β	(21)

(Continued)

TABLE 1 | Continued

Subjects	Substrates	Dose	Duration	Results	References
66 adult males	*B. bifidum* and *L. plantarum*	*Bifidobacteria*: 7.5 log CFU/g/d *Lactobacilli*: 4.59 log CFU/g/d	1 week	*Lactobacillus* and *Enterococcus* significantly increased from day 1 to day 7	(22)
180 people	*Streptococcus thermophilus* MG510 and *L. plantarum* LRCC5193	*Streptococcus thermophilus* MG510: 3.0×10^8 CFU/g/d and *L. plantarum* LRCC5193: 1.0×10^8 CFU/g/d	4 weeks	The relative abundance of *L.* plantarum remained higher in the probiotic group than in the placebo group at 8 weeks, no increment of *Streptococcus thermophilus* was observed in the faecal microbiota	(23)

IgA, immunoglobulin A; sIgA, secretory immunoglobulin A; T cells, Tregs; Th 1, T helper; IL-10, interleukin 10.

EFFECTS OF PRE-BIOTICS ON GUT MICROBIOTA AND IMMUNE FUNCTION

The concept of pre-biotics was introduced by Gibson and Roberfroid when they observed certain non-digestible oligosaccharides were selectively fermented by bifidobacteria (34). The concept of pre-biotics was firstly proposed as a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health (35). Subsequently, with the improved knowledge of gut microbiota composition and underlying mechanisms of pre-biotics (36), the definition of pre-biotics was updated in 2017 as "a substrate that is selectively utilised by host microorganisms conferring a health benefit" by an ISAPP consensus panel led by Gibson (5). Pre-biotics, such as fructooligosaccharides, galactooligosaccharides (GOS), and lactulose, have been found to significantly modulate the balance of the large intestine microorganism community by increasing the number of bifidobacteria and lactic acid bacteria (37). Other nutrients, such as pectin, cellulose, and xylan, are also beneficial to the balance of intestinal microorganisms (38). Pre-biotics play an important role in the prevention of diarrhoea (39), constipation (40), metabolic disease (41), and cancer (42) and confer positive effects on lipid metabolism (43), mineral adsorption (44), and immune regulation (45).

Effect of Pre-biotics on Gut Microbiota

Pre-biotics could regulate the balance of intestinal microorganisms and improve colon health (46–49) (**Table 2**). Inulin-type fructans (ITFs) have shown bifidogenic effects in *in vitro* and human studies (46). Inulin was found to increase bifidobacteria and lactobacilli in faecal samples from the elderly population (67, 68) and healthy adults (69) *in vitro*. In a human study, 30 obese women were recruited and randomly divided into 2 groups, one group daily received 16g ITF ($n = 15$), the other group maltodextrin ($n = 15$) for 3 months. Qualitative and quantitative analyses of faecal microbiota found that ITF intervention increased levels of *Bifidobacterium* and *Faecalibacterium prausnitzii* and decreased levels of *Bacteroides intestinalis and Bacteroides vulgate* compared to maltodextrin (50). In healthy adults, inulin could also modulate gut microbiota composition, with a significant increase in the numbers of bifidobacteria (70). Although inulin has shown modulatory impact on gut microbiota, the impact is related to the degree of polymerization (DP) of inulin. In a mice model study, high-fat-diet feeding obesity mice received inulin with DP $\leq$ 9 and DP $\geq$ 23, respectively, for 8 weeks. The results showed the inulin with longer DP could decrease the abundance of *Firmicutes* and increase the abundance of *Bacteroidetes* more significantly and perform a more beneficial impact on liver injury (71). In addition, the impact of GOS on gut microbiota was also well-studied. In a mice study, the effect of GOS in prevention and alleviation against *Escherichia coli* (*E. coli*) O157 invasion and colonisation was studied. The results showed that GOS could stimulate the growth and activities of beneficial bacteria, such as *Akkermansia*, *Ruminococcaceae*, and *Bacteroides*, and promote the production of SCFA (51). GOS could also increase the abundance of *Lactobacillus* and *Lactococcus* in rats with constipation compared to placebo, hence suppression of constipation and exerting a beneficial impact on colon health (72). In addition, GOS has also been shown to modulate gut microbiota with bifidogenic effect in volunteers with gastrointestinal symptoms, such as bloating, flatulence (73), autism (74), and those aged over 60 (67, 68). Other pre-biotics also showed a modulatory impact on gastrointestinal microorganisms. Pre-biotic Mushroom, *Bulgaria inquinans* (BI) was assessed in male C57BL/6 mice to investigate its impact on gut microbiota. Mice received BI (1 or 2%) for 4 weeks, and the results indicated the decreased diversity of gastrointestinal bacteria, increased abundance of *Faecalibaculum* and *Parabacteroides*, and decreased abundance of *Allobaculum* and *Rikenella* (52). A study showed that moderate intake of red wine polyphenols could regulate gut microbiota composition in patients with metabolic syndrome, with significantly increased the number of *Bifidobacterium*, *Lactobacillus*, and butyrate-producing bacteria (*Faecalibacterium prausnitzii* and *Roseburia*) in faeces (41). In a 100 healthy adult study, volunteers consumed placebo, 2′-O-fucosyllactose (2′FL), lacto-N-neotetraose (LNnT), or 2′FL+LNnT (2:1 mass ratio; mix) at 5, 10, or

20 g daily. The results showed that 2′FL and/or LNnT could significantly increase the relative abundance of *Actinomycetes* and *Bifidobacterium* and decrease those of *Firmicutes* and *Proteobacteria*, hence maintaining gut microbiota balance (53). Although the gastrointestinal microbiota can be modulated by pre-biotics, individual responses can vary. For example, a study aimed to investigate the impact of GOS (0.0, 2.5, 5.0, and 10.0 g GOS) on faecal microbiota of healthy human subjects found that GOS mainly increased the abundance of organisms within the *Actinobacteria*. However, only 50% of these changes can be detected by individuals. In addition, the response to the GOS varies from individual to individual (75). According to these findings, pre-biotics have been shown to modulate microbiota composition, and these effects could result in alleviating symptoms observed in patients. In addition, different pre-biotics are specific to different bacteria and their effect doses are also different. Therefore, it is important to make scientific results and conclusions reliable and develop proper instructions for consumers.

Effects of Pre-biotics on Immune Function

Protective Effect of Pre-biotics on Intestinal Epithelial Barrier

The protective effects of pre-biotics on intestinal epithelium have been confirmed, and host immunity can be enhanced by improving the integrity of intestinal epithelium (76). In a human study, 11% inulin-enriched pasta could improve the barrier function of the gut, with significantly higher levels of glucagon-like peptide-2 and lower levels of zonulin in serum, hence protecting mucosal barrier integrity and preventing gastrointestinal diseases (54). GOS could also help maintain gut barrier function and improve colon permeability. Obese adults who received 5 g/d GOS for 4 weeks showed a decreased sucralose excretion compared to placebo, suggesting improved barrier function induced by GOS (55). Similar results of GOS were also found in preterm infants (77). Although inulin and GOS could help maintain gut barrier integrity, the impacts of arabinoxylans (AX) remain controversial. In one human study, two different doses of AX (7.5 and 15 g/d) did not significantly improve gastrointestinal permeability in obese volunteers (56). Therefore, the impact of pre-biotics on the integrity and function of the intestinal epithelial barrier needs to be further assessed.

Effects of Pre-biotics on Immune Response

Pre-biotics may also contribute to the regulation of immune response through inhibiting expressions of pro-inflammatory cytokines, stimulating those of anti-inflammatory cytokines, and promoting activities of immune cells, such as macrophages, NK cells, T cells, and B cells (**Table 2**). In a porcine model study, inulin was found to induce an anti-inflammatory immune response against pathogen infection, with increased expressions of Th2-related immune genes, such as IL-13 and IL-5, and declined expressions of Th2-related immune genes, such as IFNG, IL-1α, and IL-8, which were closely related with microbiota composition (57). In another animal study, 16 SPF HLA-B27 transgenic rats were divided into two groups, the inulin group fed with the combination of chicory-derived long-chain ITFs and short-chain inulin fraction oligofructose, and the control group fed with placebo for 7 weeks. The caecum and colon tissue were collected and analysed for cytokine production. The results showed that pre-biotic intervention could reduce the levels of IL-1β and interferon-gamma (IFN-γ), suggesting the immunomodulatory impact of inulin (58). In addition, inulin could also help to regulate immune markers in patients with type 2 diabetes, with significantly declined levels of tumour necrosis factor-α (TNF-α), IL-6, and lipopolysaccharide (59). However, inulin supplementation could not regulate systemic inflammatory markers in a human study, in which twelve non-diabetic overweight adults daily received 20 g inulin for 44 days (60), the results showed that inulin had no impact on IL-8. Notably, the immunomodulatory impact of inulin depends on the DP or chain length. A double-blind, placebo-controlled human study found that DP10-60 inulin could more significantly induce the production of Th1-related cytokines and the activation of TLR2 compared to DP2-25 inulin. In addition, DP10-60 inulin could increase the numbers of B cells, Th1-cells, and the titre of anti-HBsAg, whereas DP2-25 could not (61). The impact of GOS on the immune response is also investigated. A study, which assessed the influence of Bimuno-GOS (B-GOS) on immune function of 40 elderly, found 5.5 g daily intake of B-GOS for 10 weeks could increase levels of IL-8, IL-10, and C-reactive protein, decrease levels of IL-1β, and stimulate activities of NK cells, suggesting the beneficial effects induced by B-GOS supplementation on ageing population (62). The results were in agreement with previous studies on overweight adults (78), elderly volunteers (79), and *in vitro* studies (67, 68). Not only inulin and GOS but other pre-biotics also showed immunomodulatory activity. Pre-biotic Mushroom BI was assessed in male C57BL/6 mice to investigate its impact on the host immune response. The results showed that 1 and 2% BI intervention could stimulate the proliferation of T cells from the spleen, and 2% BI could even increase IL-2 production in splenocytes, consequently influencing the peripheral and mucosal immune systems (52). In a randomised, controlled study, polyphenol supplementation reduced plasma levels of IL-8 in pre-hypertensive volunteers (63). In summary, pre-biotics could enhance host immunity in health and disease patients through protecting intestinal barrier function, activating immune cells, and regulating immune signalling pathways.

EFFECTS OF POST-BIOTICS ON GUT MICROBIOTA AND IMMUNE FUNCTION

Post-biotics include cell wall components, such as protein molecules and lipopolysaccharides, extracellular polysaccharides, and microbial metabolites of carbohydrate fermentation or protein degradation, such as SCFA and branched chain fatty acids (80). Several studies have found that post-biotics can exert positive biological functions to the host (4, 81).

TABLE 2 | Example of studies designed to determine effects of pre-biotics on gut microbiota and immune function.

Subjects	Substrates	Dose	Duration	Results	References
10 patients with metabolic syndrome and 10 healthy subjects	Polyphenols	272 mL/d	1 month	Significantly increased the number of *Bifidobacterium*, *Lactobacillus,* and butyrate-producing bacteria (*Faecalibacterium prausnitzii* and *Roseburia*) in faeces	(41)
90 men and 50 women	Ructooligosaccharides, xylooligosaccharides, polydextrose, and resistant dextrin	30 g/d	7 days	Increased serum IgG, IgM and transferrin, increased the abundance of *Bifidobacterium* and *Enterococcus*, decreased the abundance of *Bacteroides*	(42)
30 obese women	ITF	16 g/d	3 months	Increased levels of *Bifidobacterium* and *Faecalibacterium prausnitzii*, and decreased levels of *Bacteroides intestinalis and Bacteroides vulgate*	(50)
30 BALB/c mice	GOS	0.2 g GOS/100 g body weight	3 weeks	GOS could stimulate the growth and activities of beneficial bacteria, such as *Akkermansia*, *Ruminococcaceae,* and *Bacteroides*, and promote the production of SCFA	(51)
18 C57BL/6 mice	BI	1 or 2%/d	4 weeks	Decreased diversity of gastrointestinal bacteria, increased abundance of *Faecalibaculum* and *Parabacteroides*, and decreased abundance of *Allobaculum*, and *Rikenella*	(52)
100 healthy adult study	2′FL LNnT	5, 10, 20 g/d	2 weeks	2′FL and/or LNnT could significantly increase relative abundance of *Actinomycetes*, *Bifidobacterium*, and decrease those of Firmicutes and Proteobacteria	(53)
113 pre-term infants [gestational age < 32 weeks, birth weight (BW) <1,500 g]	11% inulin-enriched pasta	1.5 g/kg/d	30 days	Could improve barrier function of the gut, with significantly higher levels of glucagon-like peptide-2 and lower levels of zonulin in serum	(54)
114 Obese adults	GOS	5 g/d	4 weeks	Decreased sucralose excretion compared to placebo	(55)
47 Obese volunteers	AX	7.5 and 15 g/d	6 weeks	Did not significantly improve gastrointestinal permeability	(56)
34 crossbred Yorkshire-Landrace pigs	Inulin	10% (w/w) long-chain purified chicory inulin-enriched diet	2 weeks	Increased expressions of Th2-related immune genes, such as IL-13 and IL-5, and declined expressions of Th2-related immune genes, such as IL-1α and IL-8	(57)
16 SPF HLA-B27 transgenic rats	Inulin (5 g/kg with water)	30 ml/d	7 weeks	Could reduce the levels of IL-1β and IFN-γ, suggesting the immunomodulatory impact of inulin	(58)
52 women with type 2 diabetes	Inulin	10 g/d	8 weeks	Significantly declined levels of IL-6, and lipopolysaccharide	(59)

(Continued)

TABLE 2 | Continued

Subjects	Substrates	Dose	Duration	Results	References
12 non-diabetic overweight adults	Inulin	20 g/d	44 days	Inulin had no effect on the systemic inflammatory indexes studied	(60)
40 healthy volunteers (18–29 years)	DP10-60, DP2-25 inulin	8g/d	35 days	DP10-60 inulin could more significantly induced the production of Th1-related cytokines and the activation of TLR2 compared to DP2-25 inulin. In addition, DP10-60 inulin could increase the numbers of B cells and Th1-cells, and the titre of anti-HBsAg, whereas DP2-25 could not	(61)
40 elderly	B-GOS	5.5 g/d	10 weeks	Could increase levels of IL-8, IL-10, and C-reactive protein, decrease levels of IL-1β, and stimulate activities of NK cell	(62)
60 pre-hypertensive males	OLE (oleuropein; hydroxytyrosol)	136, 6 mg/d	6 weeks	Reduced plasma levels of IL-8 in pre-hypertensive volunteers	(63)
30 women (BMI > 30 kg/m^2, 18–65 years)	Inulin + oligofructose	16 g/d	12 weeks	Increased *B. longum, B. pseudocatenulatum,* and *B. adolescentis*	(64)
49 healthy adults	Long-chain inulin and oligofructose	50:50 mixture 8 g/d	8 weeks	NK cell activity, immunocyte phenotype bactericidal activity and T cell activity was increased	(65)
57 healthy adults	Short chain galactooligosaccharides/long chain fructooligosaccharides/pectin hydrolysate-derived acidic oligosaccharides (scGOS/lcFOS/pAOS)	15 or 30 g/d	12 weeks	Increased *Bifidobacteria*, decreased *Clostridium coccoides/Eubacterium* rectale cluster. Increased NK cell activity, reduced activation of CD14, CD25	(66)

ITF, inulin-type fructan; SCFAs, short-chain fatty acids; GOS, galactooligosaccharide; B-GOS, galacto-oligosaccharide mixture; 2′FL, 2′-O-fucosyllactose; LNnT, lacto-N-neotetraose; BI Mushroom Bulgaria inquinans; IFN-γ, interferon-gamma; AX, arabinoxylans; DP, degree of polymerization.

Effect of Post-biotic on Gut Microbiota

Post-biotic has great potential to maintain homeostasis of intestinal microbiota and improve intestinal health, through inhibiting the growth and activities of harmful bacteria and stimulating those of beneficial bacteria (**Table 3**). The impact of metabolic products of probiotic fermentation is well-studied. Cell-free spent media (CFSM) of six probiotics (*L. acidophilus* EMCC 1324, *L. helveticus* EMCC 1654, *L. plantarum ss. plantarum* EMCC 1027, *L. rhamnosus* EMCC 1105, *B. longum* EMCC 1547, and *B. bifidum* EMCC 1334) have been shown to exert strong antibacterial activity to *Escherichia coli* isolates, with inhibition zones of 11.77–23.10 mm. Notably, CFSM of *L. plantarum* had the strongest antibacterial activity compared to the other five, with a 64.57% reduction in biofilms of *E. coli* (88). In another *in vitro* study, CFSM and biofilm of probiotics *L. rhamnosus* and *L. casei* also performed an antifungal activity to *Candida albicans* (89). Similar findings were also observed in cell-free supernatant (CFS) from *L. kunkeei* against *Candida albicans* (90). CFS from *L. paracasei* CNCM I-4034, *B. breve* CNCM I-4035, and *L. rhamnosus* CNCM I-4036 isolated from a faecal sample of breast-feeding infants could suppress the growth of *E. coli*, *Salmonella,* and *Shigella* over 50%, and *L. paracasei* CNCM I-4034 CFS showed the strongest antimicrobial activity (81%) *in vivo*. However, when CFS was neutralised, decreased antimicrobial activities were observed, indicating the important role of organic acid and antimicrobial substances produced by probiotic fermentation (91). CFS from probiotic *L. rhamnosus* GG also exhibited a strong antibacterial activity against *E. coli* K1, the adhesion, invasion, and translocation of which have been shown to be blocked by CFS *in vitro*, through stimulating the production of mucin and protecting intestinal barrier function. This was demonstrated in the neonatal rat model, CFS could protect neonatal rats from *E. coli* K1 infection, through increasing expression levels of Ki67, MUC2, ZO-1, IgA, and mucin and decreasing intestinal barrier permeability (92). Besides, in a broiler model study, the impact of the mixture of CFS from *L. plantarum* RG14 and inulin on colon health and immune function was investigated. Increased numbers of total caecal microbiota and bifidobacteria, and decreased number of *Enterobacteria* and *E. coli*, were observed in birds fed with CFS and inulin compared to birds fed with placebo. In addition, CFS and inulin could stimulate the production of acetic acid

TABLE 3 | Example of studies designed to determine effects of post-biotic on gut microbiota and immune function.

Subjects	Substrates	Dose	Duration	Results	References
261 COBB 500 chicks	Inulin	1%/d	6 weeks	Increased number of total caecal microbiota and *Bifdidobacteria*, and decreased number of *Enterobacteria* and *E. coli.* In addition, CFS and inulin could stimulate the production of acetic acid in birds	(82)
12 male lambs	RG14, RG11, and TL1 from Lactobacillus plantarum	0.9%/d	60 days	Regulate barrier integrity and function in lams through increased levels of tight junction protein, occludin, claudin-1	(83)
40 C57BL mice	Post-biotic HM0539 from LGG	10 μg/d	7 days	COX-2, and iNOS, subsequently suppressing production of PGE2 and NO	(84)
300 male broilers	Post-biotics from *Saccharomyces cerevisiae* fermentation	10%/d	35 days	Decrease levels of IL-6 and IL-1ß compared to control, and increase levels of tight junction protein, occludin, and claudin-1.	(85)
Murine macrophage cell line, J774A.1 cell	EPS was isolated from *B. longum* BCRC 14634	5 μg/mL	24–48h	The proliferation of J77A.1 macrophage and their secretion of the anti-inflammatory cytokine IL-10 was elevated	(86)
Staphylococcus aureus and *Enterobacter aerogenes* of 220.25 ± 3.3 and 170.2 ± 4.6 AU/mL	CFNS of associated *Staphylococcus succinus* strain (AAS2)	Exopolysaccharide (41.3 ± 0.6 mg/L/d) and lipase production (8.3 ± 0.3 mm/d)	24h	Moderate level of exopolysaccharide and lipase production can reduce the viability of *Staphylococcus aureus* and *Escherichia aerogenes*	(66)
Ctreg isolated from 90 GF mices	SCFA production of species belonging to Clostridium cluster XI, XIV, XVII	Propionate (14–62 vs. 0.05–1.1 μmol/10^5 CFU) and acetate (118–220 vs. 0.1–2 μmol/10^5 CFU)	3 weeks	Treatment significantly increased foxp3 and IL-10 expression, this suggests that SCFA specificity induces the Treg of foxp3 and IL-10 production	(87)

CFNS, cell-free neutralised supernatant; IL-6, interleukin; COX-2, Inhibition effects on cyclooxygenase 2; iNOS, inducible nitric oxide synthase; SCFAs, short-chain fatty acids; GOS, galactooligosaccharide; PGE2, prostaglandin E2; NO, nitric oxide; IL-10, interleukin-10.

in birds, besides, the immune markers were regulated by CFS and inulin, resulting in decreased levels of IFN and TNF-α and increased levels of IL-6. The results illustrated the potential role of post-biotic CFS from *L. plantarum* RG14 in growth promoters in the poultry industry (82). CFS from *L. plantarum* RG14 could also improve the nutrients digestion and absorption in newly-weaned lambs, suggested by increasing the number of fibre degrading bacteria, such as *Fibrobacter succinogenes* and *Ruminococcus flavefaciens*, and expressions of IGF-1 and MCT-1 (93). These findings demonstrated the antimicrobial and microbiota regulatory activity of post-biotics, however, the bioactive compounds were not identified and separated.

To further explore the underlying mechanism, a few research groups focused on the exopolysaccharides (EPS), and proteins, etc. To investigate the impact of EPS on gut microbiota, an *in vitro* study applied a batch culture model and compared eleven EPS isolated from different human bifidobacteria with glucose and inulin. The results illustrated that EPS and inulin could stimulate the growth of bifidobacteria and the production of SCFA, resulting in a decreased ratio of acetic acid/propionic acid, which was contrary to glucose fermentation. In addition, the strain-specific impact of EPS was indicated, as EPS from *B. pseudocatenulatum* contributed to the growth of *Desulfovibrio* and *Faecalibacterium prausnitzii*, whereas EPS from *B. longum* contributed to the growth of *Anaerostipes*, *Prevotella*, and/or *Oscillospira* (94). The results showed EPS from bacteria could be utilised by gastrointestinal microorganisms, hence affecting colon health. The post-biotic could also inhibit the adhesion of pathogens to epithelial cells. A novel soluble protein HM0539 from *L. rhamnosus* could inhibit the adhesion and invasion of *E. coli* O157: H7 to HT-29 cell dose-dependently (95). Kaikiri et al. (96) also found that a novel gut microbiota metabolite 10-hydroxy-cis-12-octadecenoic acid (HYA) could alter the faecal microbiota community of NC/Nga mice, although the PCR-denaturing gradient gel electrophoresis analysis did not show detailed bacteria species change induced by HYA. The

impact of post-biotics on bacteria is mainly demonstrated *in vitro* and *in vivo*, hence further evidence from human clinical trials is required, and post-biotics could be used as alternative antimicrobial compounds and supplementation strategies for disease alleviation or treatment.

Effects of Post-biotics on Immune Function

Protective Effect of Post-biotics on Intestinal Epithelial Barrier

Post-biotics could modulate host immunity by improving gastrointestinal barrier function and inhibiting pathogen translocation. In one study, CFS from *L. plantarum* fermentation could regulate barrier integrity and function in lambs through increasing levels of tight junction protein, occludin, claudin-1, and CLDN-4 (83). The bioactive compound HM0539 has also been shown to stimulate mucin expression and decline expression levels of MUC2 and zonula occludens-1 (ZO-1) in the neonatal rat model, indicating its intestinal barrier protecting role (97). As shown in the above studies, it is mainly the increased tight junction protein induced by post-biotics that illustrated its positive role in the improvement of intestinal epithelial barrier function. Hence, further immunological, biochemical, and pathological section evaluation from clinical trials is warranted to support this view.

Effects of Post-biotics on Immune Response

Post-biotics may affect the innate and adaptive immune system through the interaction of many cell types along the mucosa, such as B cells, T cells, monocytes, macrophages, NK cells, and dendritic cells (DCs) (98). Cell wall components, including peptidoglycan (99), have been shown could bind to receptors on the surface of monocytes and macrophages, consequently stimulating immune cells to produce cytokines indirectly (100, 101). Tryptophan metabolites can inhibit inflammation by acting on T cell aromatics receptors and stimulating DCs to induce Treg activation through retinoic acid (102).

In addition, post-biotics could influence immune response by affecting the immune signalling pathway through modulating inflammatory cytokines. Butyrate, an important bacteria fermentation product, was shown to facilitate monocyte polarised to M2 macrophage and suppress the pro-inflammatory immune response in mice, with increased expression of Arg1 and activation of H3K9/STAT6 signalling pathway (101). Lipoteichoic acid produced by *L. plantarum* was shown to inhibit inflammation response induced by the viral pathogen in porcine intestinal epithelial cells. The results showed lipoteichoic acid could reduce levels of IL-8 and suppress ERK phosphorylation, p38 kinase, and NF-κB activation in a dose-dependent manner (103). The post-biotic HM0539 not only exhibited an inhibition effect against the pathogens but also an immunomodulatory effect. In *in vitro* cell culture and dextran sulphate sodium (DSS)-induced murine colitis model, HM0539 showed an inhibition effect on cyclooxygenase 2 (COX-2), and inducible nitric oxide synthase (iNOS), subsequently suppressing the production of prostaglandin E2 (PGE2) and nitric oxide (NO), TLR4-MyD88, and NF-κB signalling pathways, and hence might serve as a candidate strategy in inflammatory bowel disease treatment (84). Metabolic products from *Pediococcus acidilactici*, *L. reuteri*, *E. faecium*, and *L. acidophilus* fermentation were assessed in broiler chicks challenged with *Clostridium perfringens* infection. The immunomodulatory activity of these metabolic products was illustrated by the downregulating pro-inflammatory response, especially the Kyoto Encyclopaedia of Genes and Genomes (KEGG) signalling pathways (104). An animal experiment investigated the influence of post-biotics from *Saccharomyces cerevisiae* fermentation product in male broilers and found they could decrease levels of IL-6, NF-κB, and IL-1ß compared to control, and increase levels of tight junction protein, occludin, and claudin-1, indicating their anti-inflammatory activities and protective role in barrier function (85). An *in vivo* study also showed that EPS from *B. animalis* subsp. *Lactis* could modulate the immune response, inducing the downregulation of pro-inflammatory cytokines (105). In *ex vivo* cultures of mucosa from diarrhoea patients, CFS of *L. casei* DG could downregulate pro-inflammatory immune response, upregulate anti-inflammatory immune response, *via* reducing expression levels of IL-1α, IL-6, IL-8, and TLR-4, and increasing those of IL-10 (106).

The impacts of the cell wall on immune response were also compared by a few studies. The cell wall components of probiotic Ganeden *Bacillus coagulans* 30 (GBC30) were shown to stimulate the maturation of monocyte in human peripheral blood mononuclear cell (PBMC) culture. The shift of monocyte toward macrophage and DCs was observed with GBC30 cell wall components, with higher CD80 and CD86 expressions (107). Furthermore, an *in vivo* study indicated the immunomodulatory activity of GBC30 cell wall components through suppressing the production of ROS, increasing phagocytosis and migration of PBMC, enhancing the proportion of NK cells, stimulating the production of anti-inflammatory cytokine production, such as IL-4, and IL-10, and decreasing pro-inflammatory cytokine production, such as TNF-α and IFN-γ, consequently balancing Th1/Th2 immune response (86). The immunomodulatory activity of cell wall components and CFS from seventeen lactic acid bacteria strains were assessed in another *in vivo* study. The results showed that IL-10 could be stimulated by CFS of all strains in peripheral blood mononuclear cells (PMBC) culture. In addition, cell wall components and CFS could both modulate the anti-inflammatory immune response, whereas, cell wall components modulate the pro-inflammatory immune response and stimulate the activity of Tregs more strongly (108).

Autophagy plays a crucial role in the innate and adaptive immune response, regulating cell homeostasis, and modulating the renewal of cellular proteins and organelles. It has recently been found an imbalance of T cells could be caused by the deficiency in autophagy, resulting in intestinal inflammation (109). The whole peptidoglycan of *B. bifidum* showed antitumour impact in BALB/c male nude mice via increasing gene expression of bax and decreasing that of bcl-2, subsequently inducing cell apoptosis (110). Peptidoglycan expressed on *L. fermentum* BGHV110 (HV110) was isolated, and its impact on autophagy was investigated in acetaminophen (APAP)-induced hepatotoxicity in HepG2 cells. The results illustrated that activation of PINK1-dependent autophagy

could be stimulated by HV110 supplementation, which increased LC3 protein conversion and p62/SQSTM1 protein degradation (111).

These findings suggested that the immunomodulatory activity of post-biotic mainly depended on their ability to differentially regulate the production of anti-inflammatory and pro-inflammatory cytokines and the balance of Th1 and Th2. They can effectively regulate the gene expression of immune cells and the interference of transcription factors, thus driving the differentiation of the immune system. In addition, post-biotics may exert a significant effect on autophagy, hence influencing host immune response.

CONCLUSION

This review summarised the positive impacts of probiotics, pre-biotics, and post-biotics on gastrointestinal health and immune function. Several *in vitro*, *in vivo*, and clinical studies have confirmed that they play a significant role in maintaining intestinal microorganism equilibrium and regulating immune response, consequently conferring benefits to host health. Although the underlying mechanism remains to be further investigated, supplementations of probiotics, pre-biotics, and post-biotics could lower colonic pH, produce antibacterial molecules, stimulate the growth and activities of beneficial bacteria, and suppress the growth of pathogens. In addition, they also play an important role in the modulation of host immunity, via improving gut barrier function, preventing pathogen translocation, altering pattern recognition receptor expression, modulating crucial signalling pathways, stimulating immune cell activities, and balancing Th1/Th2 immune response. Notably, the efficacies of probiotics, pre-, and post-biotics are typically dose-dependent, especially exhibiting species-specific for probiotic strains and structure-activity relationships for pre- and post-biotics. In addition, the profile of host microorganisms and immunity may differ based on the age, gender, exercise, health conditions, geography, etc, of the host, hence, it is necessary to consider these factors and assess increasingly quantitative and qualitative impacts of pre-biotics, probiotics, and post-biotics on gut microbiota and immune system. Besides, the underlying mechanisms should be fully illustrated via combining *in vitro*, *in vivo*, and clinical studies and biochemical evaluations.

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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