PROBIOTICS AND ITS EFFECTS ON INFLAMMATORY AND INFECTIOUS DISORDERS

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PROBIOTICS AND ITS EFFECTS ON INFLAMMATORY AND INFECTIOUS DISORDERS

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EDITORIAL
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Editorial: Probiotics and its Effects on Inflammatory and Infectious Disorders

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Editorial on the Research Topic

Probiotics and its Effects on Inflammatory and Infectious Disorders

INTRODUCTION

Human beings have evolved surrounded by a variety of microorganisms. Aside from their presence as outer neighbors, bacteria, viruses, fungi, archaea and parasites, may also live in close proximity inhabiting distinct ecological niches in our body, and are known as microbiota (Simon et al., 2019). For decades, these "foreigners" were believed to have only very specific and limited roles towards the functionality of human biological systems. However, especially with the use of next generation sequencing (NGS), mainly targeting the largest population of living microorganisms inhabiting different body sites, which are bacteria, our knowledge in this field has drastically increased (Toju et al., 2020).

Currently, the microbiota of different body sites is believed to influence (or to be influenced by) distinct biological systems, including nervous, immune and endocrine, among others, thus directly contributing to the maintenance of healthy and disease states. Among these different microbial niches, the gut is by far, the richest (in diversity and composition) and the most explored in different contexts (Chen et al., 2018). However, it is not clear yet if microbial disturbances (known as dysbiosis) are a consequence or the causative agent of inflammatory and infectious diseases. Despite the ongoing advances in exploring microbial communities in the aforementioned scenarios, the therapeutic potential of microorganisms have been explored for centuries in human culture. Either as fermented foods (with unknown amounts and composition of microorganisms) or using known species of microbes at specific concentrations, such approach aims at modulating microbial composition and diversity, mainly gut microbiota, thus reestablishing microbial balance and constraining inflammation. Although some beneficial properties of probiotics have been explored in different contexts (Sales-Campos et al., 2019), the ongoing amount of research identifying the therapeutic potential of known/unknown microorganisms, used alone or in combination with classical therapies, may represent a new frontier in the field of microbiota manipulation, thus leading to a more favorable outcome of infectious and inflammatory diseases.

PROBIOTICS IN INFLAMMATORY DISORDERS

The mutual contribution between microbiota, mainly from Gastrointestinal tract (GIT), and different biological systems dictates the outcome of health and disease. In this view, Curciarello

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et al. investigate the potential anti-inflammatory effect of *Lactobacillus kefiri* in patients with Inflammatory Bowel Disease (IBD). More specifically, *L. kefiri* reduced the release of IL-6 and IL-8 from inflamed biopsies. Also, for the first time, they have reported the immunomodulatory effect of a kefir-isolated strain, *L. kefiri* CIDCA 8,348, on human intestinal tissue and primary T cells from IBD patients. Additionally, Belo et al. investigated the potential role of surface-layer proteins (Slp), notably SlpB from *Propionibacterium freudenreichii* CIRM-BIA 129, as a modulator of inflammation in Ulcerative colitis (UC). Mice exposed to DSS and treated with the probiotic *Lactococcus lactis* NCDO 2118 expressing a recombinant SlpB, had reduced severity of colitis and improved disease score, when compared to untreated mice. Also, it constrained inflammation in diseased mice. Finally, Savassi et al. developed a lyophilized synbiotic, to address its effects as adjuvant treatment in mucositis. The formulation reduced weight loss, intestinal permeability, and the intensity of inflammation in the duodenum, ileum, and colon; besides, it decreased the levels of pro-inflammatory cytokines. These data suggest probiotic bacteria as promising candidates for the treatment and prevention of GIT inflammatory diseases.

Strategies aiming at modulating the microbiota-gut-brain axis may represent a new frontier in developing therapeutic approaches for neuropsychiatric disorders. Wang et al. explored the role of the psychobiotic *Lactobacillus johnsonii* BS15 on the gut-brain axis to elucidate whether it could modulate the gut environment, thus, preventing memory dysfunction in an experimental model of psychological stress. The psychobiotic not only enhanced the performance of mice under stressing conditions, but also positively modulated the hypothalamic–pituitary–adrenal axis and memory-related functional proteins, besides maintaining gut barrier integrity.

In general, autoimmune or immune-mediated diseases are multifactorial disorders in which genetic mutations, environmental factors, immune imbalance and microbiota dysbiosis contribute to disease onset. Guo et al., reviewed the beneficial effects of probiotics in experimental models of Systemic Lupus Erythematosus (SLE) highlighting its impact towards a reduction in cardiovascular and renal complications. If the number of human studies addressing the role of probiotics in SLE is limited, Ferro et al., showed a completely different scenario for rheumatoid arthritis (RA). The probiotic bacteria *Lactobacillus casei* seems to represent the best candidate for application as adjuvant therapy for RA patients. In addition, Pagnini et al., discussed the potential role of probiotics on modulating the vitamin D pathway to treat IBD.

Obesity is marked by a low grade chronic inflammation and dysbiosis, and the review submitted by Maioli et al., explores the role of *Faecalibacterium prausnitzii* as a potential treatment and a putative biomarker in this scenario.

Liver inflammation, as a consequence of alcohol consumption, can evolve to more severe forms of liver damage, including cirrhosis and hepatocarcinoma, where such disturbances can be generically described as alcoholic liver disease (ALD). Also, ALD outcome is drastically influenced by gut dysbiosis which suggests a role for probiotics, in combination with classic approaches, to attenuate liver inflammation and ameliorate disease progression, as reviewed by Fuenzalida et al.

PROBIOTICS IN INFECTIOUS DISORDERS

Members of the genus Bifidobacterium are the first to colonize the human gut, exerting health benefits for the host, and are also ubiquitously used as probiotics. Shimabukuro et al. evaluated the effect of two Bifidobacterium strains in inhibiting *Porphyromonas gingivalis* interaction with host cells and biofilm formation in periodontitis. More specifically, *Bifidobacterium bifidum* 1622A showed greater potential to control periodontitis, once it has not changed the inflammatory parameters significantly and prevented alveolar bone loss.

Cell-free supernatants of probiotic bacteria have been proposed lately as a safer option when compared to the use of live bacteria. Dubey et al. explored the use of cell-free supernatant of *Lactiplantibacillus plantarum* MTCC 2621 (Lp2621) to evaluate the potential antibacterial, hemolytic, antioxidant and wound healing properties using *in vitro* and *in vivo* approaches. Treatment with Lp2621 gel upregulated IL-6 in the early phase of wound healing and enhanced IL-10 expression in the later phase. Also, this treatment improved angiogenesis, proliferation of fibroblasts, re-epithelization, and recruitment of polymorphonuclear leukocytes.

Probiotics have also been used to treat other infectious disorders. The crosstalk between gut and lungs has been proposed as a key driver for host homeostasis. For this reason, gut microbiota dysbiosis also impacts lung function, thus increasing the susceptibility of respiratory tract infections. In this regard, Cruz et al. reviewed the role of prebiotics, probiotics and synbiotics on the prevention or as therapeutic approaches to treat bacterial, viral, fungal and helminthic infections affecting lungs. In this context, and based on the limitations concerning anthelmintic drugs, Saracino et al., highlighted the effects of probiotics to elicit a type 2 immune response, and therefore, improving the response and control against helminthic infection.

CONCLUSION

During the past years, a growing number of researchers have been dedicating time and efforts to explore the role of probiotics in different scenarios. Likewise, the popularization of NGS drastically improved our knowledge about microbiota composition and ecology, which not only facilitated the identification of microorganisms with probiotic potential but also the improvement of already known species. Despite this promising scenario, the use of probiotics, especially in clinical practice, remains strict to only a few inflammatory diseases, mainly those affecting GIT. Unfortunately, the number of human studies addressing the role of probiotics in infectious diseases is even smaller. However, the rising number of experimental studies addressing the beneficial impact of probiotics, mainly as adjuvant therapy, and the association

between its use and the reestablishment of gut microbial balance, may encourage a broader use by clinicians in the aforementioned scenarios.

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SCS and HS-C wrote and drafted the whole editorial.

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BRIEF RESEARCH REPORT
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Probiotic Lactobacilli Isolated from Kefir Promote Down-Regulation of Inflammatory Lamina Propria T Cells from Patients with Active IBD

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Ulcerative colitis and Crohn's disease, the two main forms of inflammatory bowel disease (IBD), are immunologically mediated disorders. Several therapies are focused on activated T cells as key targets. Although *Lactobacillus kefiri* has shown anti-inflammatory effects in animal models, few studies were done using human mucosal T cells. The aim of this work was to investigate the immunomodulatory effects of this bacterium on intestinal T cells from patients with active IBD. Mucosal biopsies and surgical samples from IBD adult patients (n = 19) or healthy donors (HC; n = 5) were used. Lamina propria mononuclear cells were isolated by enzymatic tissue digestion, and entero-adhesive *Escherichia coli*-specific lamina propria T cells (LPTC) were expanded. The immunomodulatory properties of *L. kefiri* CIDCA 8348 strain were evaluated on biopsies and on anti-CD3/CD28-activated LPTC. Secreted cytokines were quantified by ELISA, and cell proliferation and viability were assessed by flow cytometry. We found that *L. kefiri* reduced spontaneous release of IL-6 and IL-8 from inflamed biopsies *ex vivo.* Activated LPTC from IBD patients showed low proliferative rates and reduced secretion of TNF-α, IL-6, IFN-γ and IL-13 in the presence of *L. kefiri*. In addition, *L. kefiri* induced an increased frequency of $CD4^+FOXP3^+$ LPTC along with high levels of IL-10. This is the first report showing an immunomodulatory effect of *L. kefiri* CIDCA 8348 on human intestinal cells from IBD patients. Understanding the mechanisms of interaction between probiotics and immune mucosal cells may open new avenues for treatment and prevention of IBD.

Keywords: ulcerative colitis, Crohn's disease, probiotics, immunomodulation, mucosal samples, *Lactobacillus kefiri* CIDCA 8348

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INTRODUCTION

Inflammatory bowel disease (IBD) comprises a complex group of chronic relapsing diseases among which the most conspicuous are ulcerative colitis (UC) and Crohn's disease (CD). Patients with IBD suffer chronic inflammation of the bowel mucosa that may affect the mucosal layer (UC) or the whole bowel wall (CD) (Torres et al., 2017; Ungaro et al., 2017). The etiology of these diseases is largely unknown, although factors such as diet, certain genes related to the sensing of luminal microbes, secretion of antimicrobial peptides and autophagy have shown to be associated (J. S. Lee et al., 2021; Kaser and Blumberg 2011). Changes in microbiota composition are typically observed in IBD patients. These findings, along with results obtained from animal models, including the fact that germ free mice do not develop experimental colitis, highlight the impact of the microbiota composition in the pathogenesis of these disorders (Onderdonk et al., 1977).

Metagenomic strategies have revealed an altered gut microbial composition in IBD patients compared to healthy subjects, known as dysbiosis (Liu et al., 2020). IBD patients usually present a reduced bacterial diversity, with low levels of Firmicutes and Bacteroidetes and increased levels of facultative anaerobic Proteobacteria and Bacilli. However, the implication of these findings for pathogenesis is not clear. Also, persistent bacterial infection by enteric bacteria, such as adherent-invasive *Escherichia coli*, has been observed (Abdelhalim et al., 2020; J. G. Lee et al., 2019). Intensive research is being carried out to determine whether changes in microbiota are causative or a consequence of the chronic inflammation observed in IBD. Inflammation probably comes from a sum of effects: an increased amount of mucosal associated bacteria, along with high intestinal permeability also present in these patients suggest that bacteria could penetrate the epithelial barrier, thus contributing to inflammation. These microorganisms in turn may promote the release of pro-inflammatory factors such as TNF-α, which boost inflammation (Shawki and McCole, 2017). Altered *trans*-cellular and *para*-cellular permeability have been described in IBD, evidenced by the presence of intracellular bacteria inside epithelial cells, and by modified tight junction protein expression and increased myosin light chain kinase (MLCK) activity (Yu Chia-Hui, 2018). Moreover, gut permeability can be influenced by changes in metabolites produced by the microbiota (Schlegel et al., 2020; J. S. Lee et al., 2021). The healthy gut microbiome produces bioactive metabolites, including short chain fatty acids (SCFA), which contribute to intestinal homeostasis and epithelial cell nutrition (Postler et al., 2017). Diminished levels of these molecules could favor an impaired barrier function and inflammatory environment.

The role of $CD4^+$ T lymphocytes is critical in IBD. In CD, IL-12 signaling induces the differentiation of $CD4^+$ T cells into IFN-γ secreting cells, while IL-23 contributes to the differentiation of $CD4^+$ T cells into Th1 and Th17, thus increasing IFN-γ and IL-17 secretion, respectively. IL-6, IL-23 and TGF-β also participate in CD pathogenesis. In UC, $CD4^+$ T lymphocytes also secrete IL-4 and IL-13, which contribute to tissue damage (Zenewicz et al., 2009). In addition, activated $CD4^+$ T cells have increased proliferation rate and are resistant to apoptosis in these pathologies (Schmitt et al., 2019). Anti-TNF-α therapies target these activated T cells, inducing T cell apoptosis, but unfortunately, many patients do not respond or become refractory within years of treatment, and require surgery (Yanai and Hanauer, 2011). Consequently, great effort is being made to develop new therapies for IBD, aimed to modulate T cell response.

Probiotics were defined several years ago as "live microorganisms that confer a health benefit to the host when administered in adequate amounts" (Food and Agriculture Organization and of the United Nations/World Health Organization, 2002). Recently, the term "postbiotic" has come to be used and refers to the functional bioactive compounds generated during microbial fermentation processes, including extracellular polysaccharides, (SCFA) and different microbial cell components, which can have beneficial effects on host health (Wegh et al., 2019). Probiotics have been studied as having beneficial properties in murine and rat models of colitis. Different strains of *Bifidobacterium*, *E. coli* and *Lactobacillus* have shown anti-inflammatory effects (Jakubczyk et al., 2020). Even though data from probiotics treatment in patients are controversial, evidence of their usefulness in combination with pharmacological treatments is arising (Shanahan and Quigley, 2014; Qiao et al., 2016; Fan et al., 2019; Ahn et al., 2020). Nevertheless, basic studies regarding their effect on IBD lamina propria cells are scarce. Kefir is an ancient product traditionally obtained by fermentation of milk with kefir grains, and many health-promoting properties have been associated with its consumption (Farag et al., 2020). Kefir grains are composed of different species of bacteria and yeasts that live symbiotically in a complex matrix constituted by proteins and polysaccharides (Bengoa et al., 2019). *Lactobacillus kefiri* is one of the most important lactobacilli retrieved from kefir, with reported quantification of around 10^8 bacteria/mL in fermented milk (Garrote et al., 2005). Most *L. kefiri* strains are safe for consumption and resistant to the harsh conditions of the gastrointestinal tract. Moreover, different beneficial effects, including immunomodulation and prevention of metabolic disorders, have been reported for these species (Slattery et al., 2019). In particular, the kefir-isolated strain *L. kefiri* CIDCA 8348 has shown to be sensitive to several antibiotics, to lack virulence factors, and to be safe for oral consumption in mice (Carasi et al., 2014). Noteworthy, mice orally treated with this strain showed reduced expression of pro-inflammatory molecules and an up-regulation of anti-inflammatory mediators, as well as secretory IgA and mucins in the gut (Carasi et al., 2015). Moreover, it was reported that administration of *L. kefiri* CIDCA 8348 prevents the deleterious effects of a fructose-rich diet in a murine model, exerting an anti-inflammatory activity in the adipose tissue (Zubiría et al., 2017).

In this work, we studied the immunomodulatory properties of *L. kefiri* CIDCA 8348 on $CD4^+$ T lymphocytes from the lamina propria of IBD patients. This *Lactobacillus* strain diminishes the proliferation of these cells and the secretion of pro-inflammatory cytokines through an NF-κB dependent pathway.

TABLE 1 | Clinical features of healthy control (HC) and IBD patient groups.

	HC group	IBD group	
		UC	CD
Number of patients	5	11	8
Sex (*n* = ***patients***)	Female = 3, Male = 2	Female = 6, Male = 5	Female = 7, Male = 1
Age of the patients [average (range)]	51 (23–73) y	39 (18–64) y	38 (21–59) y
Site of sampling (*n= patients*)[a]	Left colon (2) rectum (3)	Cecum (1), right (2), left (4), transverse (2), sigmoid (3), rectum (8)	Cecum (2), right (3), left (2), transverse (1), rectum (2)
Endoscopic activity at sampling time (*n= patients*)	No activity = 5	mild = 6, moderate = 3 severe = 2	No activity = 1[b], mild = 1, moderate = 4 severe = 2
Treatment at sampling time (*n= patients*)[c]	Not applicable	Adalimumab (1) corticosteroids (3) mesalazine (9) azatioprine (4)	Adalimumab (2) infliximab (1) corticosteroids (4) mesalazine (3) azatioprine (2)

UC = ulcerative colitis, CD = Crohn's disease, age is expressed in years (y).
[a]*Samples were taken from more than one site in some patients.*
[b]Uninflamed samples were only used for LPTC in vitro assays.
[c]*Some patients were under multiple pharmacological treatment.*

MATERIALS AND METHODS

Patients and Tissue

Surgical samples of colon or rectum from patients who underwent partial or total colectomy, and endoscopic colonic biopsies were taken from macroscopically inflamed mucosa of IBD adult patients affected by CD (n = 8) or UC (n = 11). The diagnosis was made according to clinical, endoscopic and histological criteria. The extent and location of the UC and CD were evaluated during colonoscopy. Clinical activity in UC was evaluated by total Mayo score (inactive ≤2, mild activity three to five, moderate seven to nine, severe 10–12) and by Harvey-Bradshaw Index (Inactive score <5; mild activity ≥5, moderate ≥7, severe ≥16) in CD (**Table 1**) (Peyrin-Biroulet et al., 2016). In addition, mucosal samples were collected endoscopically from the colon of adult subjects who were neither diagnosed with IBD nor any other inflammatory condition of the gut. Samples of healthy mucosa were obtained from surgical specimens of colorectal cancer partial colectomies. These specimens constituted the "healthy control patient" samples (HC, n = 5). The local Ethics Committee (*Comité de Ética en Investigaciones, Hospital de Gastroenterología Carlo B. Udaondo, Ciudad Autónoma de Buenos Aires,* Res. 07–07–2016) approved the protocols and informed written consent was obtained from every patient.

Bacteria and Conditioned Medium

Lactobacillus kefiri CIDCA 8348 belonging to the collection of the Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA, CONICET-UNLP-CIC, Argentina) was used. The strain was cultured in deMan-Rogosa-Sharpe (MRS) broth (Difco, Beauvais, France) at 37°C for 48 h in aerobic conditions. Bacteria were harvested, washed twice and finally resuspended in sterile phosphate-buffered saline (PBS) at OD^{550} = 2.0 (approx. 1–2x10^8 cfu/ml). In order to prepare the *L. kefiri*-conditioned medium (CM), bacterial suspension at OD^{550} = 0.1 in Ultraculture medium was incubated for 24 h at 37°C and 5% CO_2. Then, bacteria were removed by centrifugation and supernatant was collected and stored at −20°C until used. Entero-adhesive (EA) *E. coli* was grown in Luria Bertani Broth to OD^{550} = 0.8. Cells were then harvested by centrifugation and resuspended in sterile PBS. Extracts were sonicated for 10 pulses at 100% and centrifuged al 10.000×g for 15 min. Protein concentration of the resulting solution was assayed by bicinchoninic acid assay (Pierce, Thermo Fisher Scientific, Rockford, IL, United States) and stored at −20°C. Prior to use, extracts were thawed and diluted in fresh medium to the desired concentration.

Organ Cultures

Endoscopic mucosal biopsies from control subjects (n = 5) or IBD patients (n = 13) were placed (one biopsy per well) in 24-well plates. Individual biopsies were cultured in 300 µL of serum-free RPMI 1640 medium (Gibco, Thermo Fisher Scientific, Rockford, IL, United States), supplemented with 100 U/mL penicillin and 100 µg/ml streptomycin, and cultured at 37°C, 5% CO_2, with or without probiotics and/or 10 ng/ml TNF-α. After 24 h of *ex vivo* culture, supernatants of mucosal biopsies were collected and stored at −70°C until used.

Lamina Propria Mononuclear Cell Isolation

The mucosa layer of surgical pieces was mechanically separated from the full-thickness surgical specimen. The epithelial layer was removed with 1 mmol/L ethylenediaminetetraacetic acid (EDTA) and 1 mmol/L dithiothreitol (DTT) in 1 mM HBSS (Gibco, Thermo Fisher Scientific, Rockford, IL, United States). After stirring for 1 h at 37°C, the supernatant was removed. The remaining tissue was minced with a scalpel and digested with type 1 A collagenase (1 mg/ml; Sigma-Aldrich, St. Louis, MO, United States) and DNAse (10 IU/ml, Roche, Thermo Fisher Scientific, Rockford, IL, United States) in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/ml streptomycin for 2 h with stirring at 37°C and 5% CO_2. Biopsies were washed three times in HBSS containing EDTA and DTT with stirring and then digested as described above. Cells were filtered through 40 µm cell-strainers (Becton

Dickinson, Franklin Lakes, NJ, United States) and washed with RPMI-1640 medium containing 10% FBS, 100 U/mL penicillin, and 100 μg/ml streptomycin.

Generation of Lamina Propria T-Cell Lines (LPTC)

LPMC were washed and cultured in 300 μL of serum-free Ultraculture medium (LONZA, Basel, Switzerland), supplemented with 2 mM glutamine, 20 μM 2-mercaptoethanol, and Antibiotic-Antimicotic (Gibco, Thermo Fisher Scientific, Rockford, IL, United States). In order to obtain specific LPTC, cells were stimulated with entero-adhesive *E. coli* extracts (0.5 μg/ml) at 37°C, 5% CO_2. Cultures without *E. coli* extract served as controls. Four days later, cells were treated with recombinant human (rh) IL-2 (10 U/mL, Preprotech, Rocky Hill, NJ, United States), rhIL-7 (10 ng/ml, Preprotech, NJ, United States) and rhIL-15 (10 ng/ml, Preprotech, Rocky Hill, NJ, United States) as reported by Rabinowitz et al. (2013). After 5 days, viable T cell blasts were enriched by Ficoll-Paque ™ (GE, Healthcare, Life Sciences, Danderyd, Sweden) gradient and then incubated in 96-well round-bottom cultures plates, in Ultraculture medium, supplemented with the cytokines mentioned above, 10% human AB^+ plasma and irradiated peripheral blood mononuclear cells PBMC (1×10^5) (Bohle et al., 2003). Cells were expanded in this same medium twice a week until enough cells were obtained, thus generating EA *E. coli* specific LPTC (from now on LPTC).

LPTC Cultures and Proliferation Assays

After expansion, LPTC were rested without further feeding for 10 days. Cells were washed, labeled with CFSE (Sigma-Aldrich, St. Louis, MO, United States) proliferation dye and stimulated with human anti-CD3 and anti-CD28 antibodies (1 μg/ml, eBioscience, San Diego, CA, United States), *L. kefiri* (2:1 bacteria:eukaryotic cell relation), *L. kefiri* conditioned media or combinations thereof. EA *E. coli* cultures were used as a positive control, also in a 2:1 relation to lymphocytes. Negative controls without the addition of stimuli were included. Assays were performed in serum-free AIMV medium (Thermo Fisher Scientific, Rockford, IL, United States). After 4 days, culture supernatants were harvested for cytokine evaluation and cells were stained with anti-CD4-APC (BD Pharmingen, San Diego, CA, United States), 7-AAD (BD Pharmingen, San Diego, CA, United States) and flow cytometry was performed using a FACS CALIBUR (BD, Franklin Lakes, NJ, United States). Flow cytometry data from two independent experiments from each patient was analyzed using FlowJo software (BD, Franklin Lakes, NJ, United States).

For intracellular FOXP3 staining, cells were stimulated as previously indicated. After 4 days cells were harvested and stained with anti-CD4-APC (BD Pharmingen, San Diego, CA, United States). Cells were then treated with BD Cytofix/Cytoperm Fixation/Permeablization Kit (BD Pharmingen, San Diego, CA, United States) and stained with anti-FOXP3-PE (BD Pharmingen, San Diego, CA, United States). Events were acquired with a FACS CALIBUR (BD, Franklin Lakes, NJ, United States). Lymphocytes were gated in the FSC/SSC scatter plot. $CD4^+$ lymphocytes were then selected from this gate and analyzed for FOXP3 staining. The negative threshold was set using fluorescence minus one controls (FMO). Duplicate independent experiments were performed for each patient.

ELISA

Cytokines were quantified in organ culture and LPTC supernatants (each sample was tested in duplicate), following manufacturer's instructions: human IL-6 (R&D systems, Minneapolis, MN, United States), human IL-8 (BD, Franklin Lakes, NJ, United States), human IFN-γ and human TNF-α (ImmunoTools, Friesoythe, Germany), human IL-13 (Invitrogen, Thermo Fisher Scientific, Rockford, IL, United States), human IL-10 (R&D systems, Minneapolis, MN, United States), human IL-1β and human IL-17 A (Biolegend, San Diego, CA, United States).

Western Blot

Protein extracts from LPTC incubated with anti-CD3/anti-CD28 alone or combined with *L. kefiri* for 30 min were used. Briefly, cells were harvested and lyzed with RIPA buffer (Sigma-Aldrich, St. Louis, MO, United States) in the presence of a protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO, United States). Protein content was quantified by bicinchoninic acid assay (Pierce, Thermo Fisher Scientific, Rockford, IL, United States) and extracts were stored at −80°C until use. Protein samples were resolved on 10% SDS-PAGE under reducing conditions (BioRad Mini-Protean III; BioRad, Hercules, CA, United States), and transferred onto a nitrocellulose membrane for 1 h at 300 mA. Blots were blocked and probed with a rabbit anti-p65 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, United States), followed by the appropriate HRP-conjugated secondary antibody (BioRad, Hercules, CA, United States). Protein bands were visualized by enhanced chemiluminescence (ECL Plus; GE Healthcare, Danderyd, Sweden) according to the manufacturer's instructions. Blots were stripped and incubated with a rabbit anti-human β-actin antibody (Abcam, Cambridge, MA, United States) diluted 1:3,000, as an internal loading control. The bands were scanned with C digit scanner (LI-COR Biosciences, Lincoln, Nebraska, United States) and quantified using ImageJ software.

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism eight software (GraphPad Software, San Diego, CA, United States). The significance of the difference was determined using an independent-sample *t*-test or 1-way ANOVA after visual inspections of distribution using Q–Q plots and Shapiro–Wilk normality test analysis. In cases when data did not adjust to normal distribution, Wilcoxon matched paired test or Friedman statistics were applied. A p-value < 0.05 was considered statistically significant.

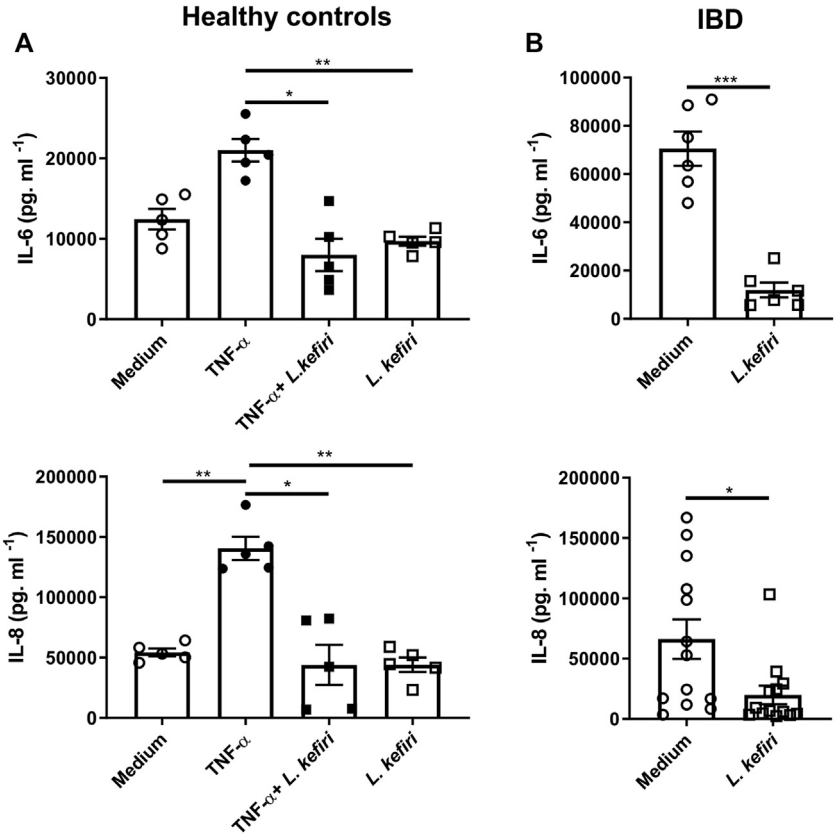

FIGURE 1 | *Lactobacillus kefiri* modulates the secretion of IL-6 and IL-8 in organ culture **(A).** Biopsies from healthy donors ($n = 5$) were incubated o. n. with TNF-α, *L. kefiri* or a combination of both **(B)**. Biopsies form IBD patients ($n = 13$) were incubated with or without *L. kefiri.* Supernatants were collected and cytokines were assessed by ELISA. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Ethics Statement

The studies involving human participants were reviewed and approved by Ethics Committee of the Hospital de Gastroenterología Carlos B. Udaondo. The patients/participants provided their written informed consent to participate in this study.

RESULTS

Lactobacillus Kefiri Reduces the Pro-Inflammatory Cytokine Secretion in Inflamed Biopsies from IBD Patients

In order to evaluate the immunomodulatory effects of *L. kefiri* CIDCA 8348, we assayed IL-6 and IL-8 in culture supernatants of tissue explants from healthy subjects ($n = 5$) incubated with the pro-inflammatory stimulus TNF-α, with or without probiotics (**Figure 1A**). TNF-α promoted the secretion of IL-8 ($p < 0.01$), and a trend in IL-6 secretion ($p = 0.0616$) with respect to unstimulated healthy tissue. Explants exposed to *L. kefiri* produced reduced levels of these TNF-α -induced cytokines ($p < 0.05$). We then studied the effect of the bacteria on biopsies from inflamed mucosa of IBD patients ($n = 13$). *L. kefiri* significantly dampened the spontaneous release of pro-inflammatory cytokines *ex vivo* ($p < 0.001$) for IL-6 and IL-8 ($p < 0.05$) (**Figure 1B**). A significant similar suppression of IL-1β and IL-17 A secretion was observed in IBD samples ($p < 0.01$) (**Supplementary Figure S1**).

Lactobacillus Kefiri Modulates the Cell Proliferation of Stimulated Microbiota-Specific Lamina Propria T Lymphocytes

Since EA *E. coli* is overrepresented in the microbiota of IBD patients, we expanded *E coli*-specific LPTC. We could not retrieve LPTC from HC since these cells did not survive long *in vitro* under EA *E. coli* extract stimulation. We therefore proceeded to generate LPTC from 17 colon samples from patients with active IBD. We found that EA *E. coli* extracts significantly increased ($p < 0.01$) the proliferation of LPTC from IBD patients (**Figure 2A**).

Aiming to study whether *L. kefiri* modulates IBD LPTC, anti-CD3/CD28-stimulated cells were co-incubated with the probiotics. We found that the increased proliferation of LPTC ($p < 0.01$) and $CD4^+$ LPTC ($p < 0.001$) (**Figures 2A–C**) were significantly inhibited by incubation with *L. kefiri* ($p < 0.01$ and $p < 0.05$, respectively). Also, the cell viability remained unchanged in all conditions, as shown with 7AAD staining (**Figure 2D**). *E. coli* used as control promoted cell proliferation, thus

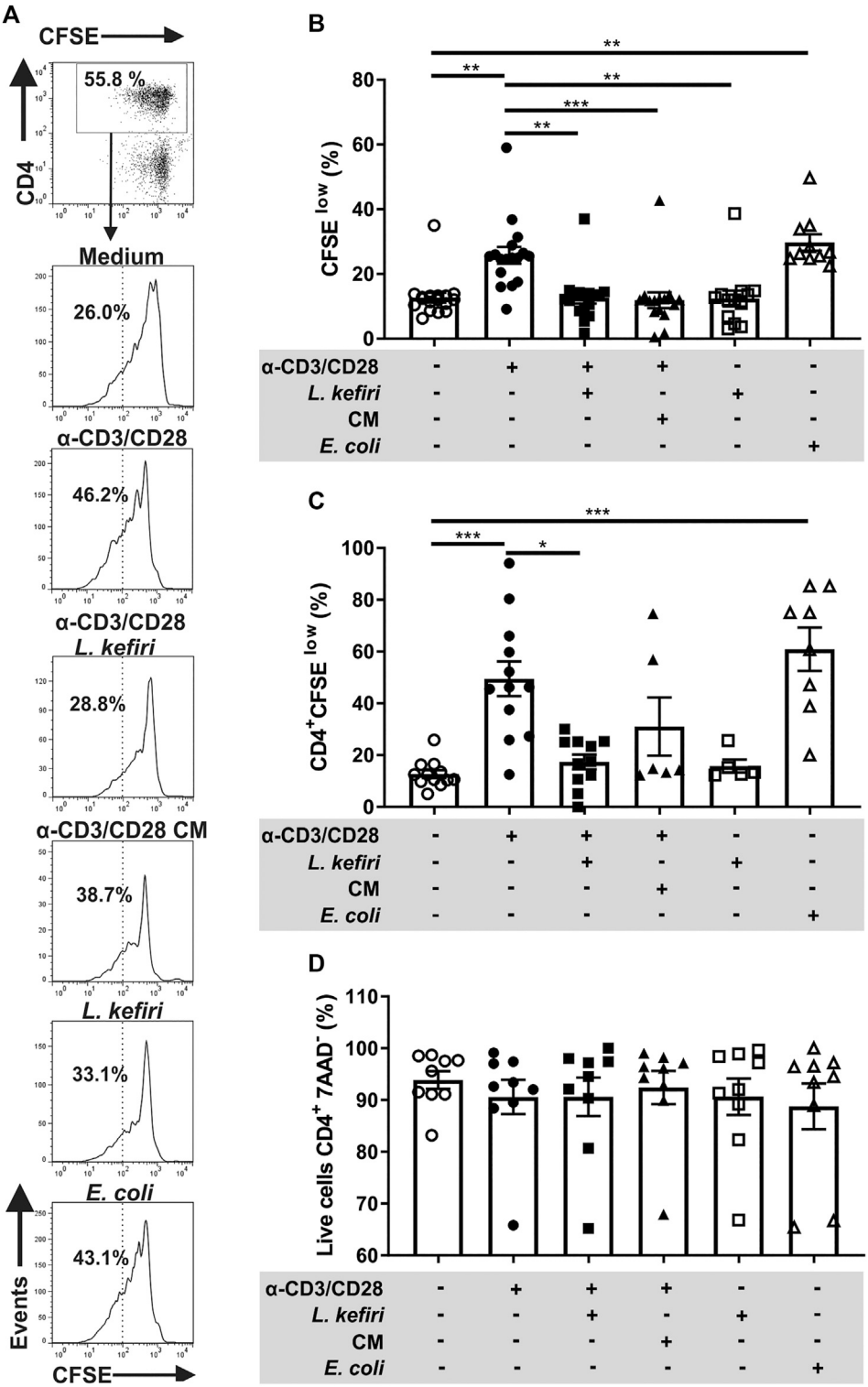

FIGURE 2 | Proliferation and LPTC response are impaired by *L. kefiri* **(A).** LPTC were activated with anti-CD3 and anti-CD28 and incubated with live probiotic, conditioned medium (CM), EA ***E. coli*** or medium. Cell proliferation was assessed by flow cytometry with CFSE. The gating strategy is shown: $CD4^+$ cells were gated from the lymphocyte gate on the forward and side scatter plot. Histograms for CFSE staining are shown separately for each stimulus. Representative results from one patient are shown **(B)**. LPTC proliferation shown as percentage of "CFSE low" staining population under each stimulus *in vitro.* Each symbol represents one independent experiment **(C)**. Proliferation of $CD4^+$ LPTC is shown as percentage of CFSE low cells obtained by the strategy shown in A, each symbol represents one independent experiment **(D)**. $CD4^+$ LPTC cell death was assessed by flow cytometry with 7-AAD staining, in the same LPTC cultures. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

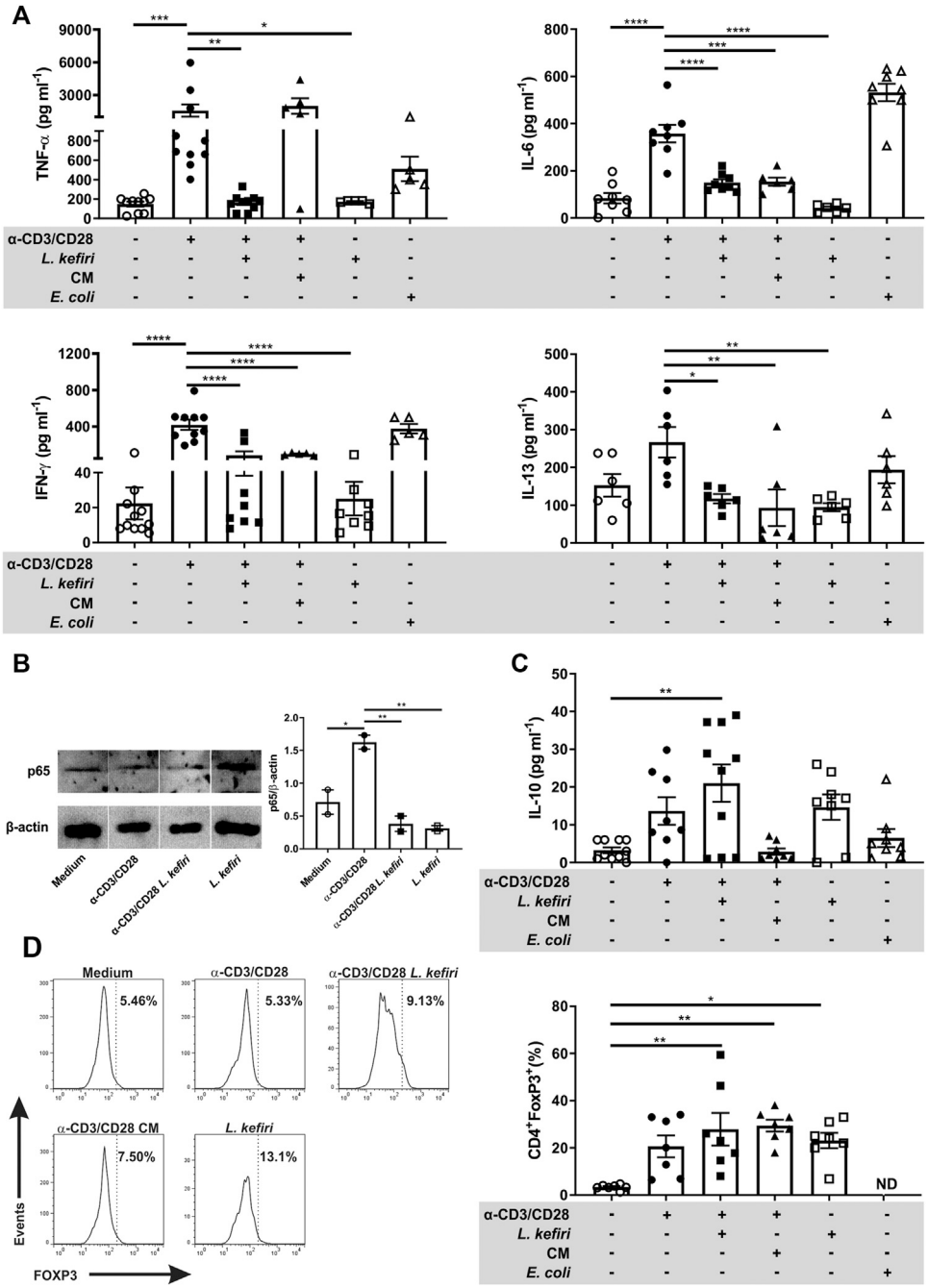

FIGURE 3 | *Lactobacillus kefiri* modulates LPTC cytokine secretion, FOXP3 expression and NF-κB signaling **(A).** Effect of *L. kefiri* and CM on cytokine secretion from activated LPTC. Cytokines were assessed by ELISA **(B)**. Immunoblots of LPTC protein extracts after 30 min of stimulation (representative of two independent assays) and statistical analysis of intensities of bands corresponding to p65 **(C)**. Quantification of IL-10 by ELISA in supernatants of the same LPTC assays shown in **A (D).** Representative histograms of the flow cytometry data analysis of LPTC. Frequency of $CD4^+FOXP3^+$ T cells after 4 days incubation with live probiotic or CM. $FOXP3^+$ cells were evaluated in $CD4^+$ cells from the lymphocytes gate. Anti-CD3 and anti-CD28 antibodies were used for T cell activation. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

confirming the specificity of T cells ($p < 0.01$ and $p < 0.001$, respectively).

Considering that lactic acid bacteria secrete SCFA with immunomodulatory properties, we also incubated LPTC with conditioned medium from *L. kefiri*. As depicted, we found that LPTC proliferation was significantly suppressed ($p < 0.001$), whereas for activated-$CD4^+$ T cells, it did not reach statistical significance ($p = 0.106$) (**Figures 2A–D**).

Lactobacillus Kefiri Ameliorates the Pro-Inflammatory Cytokine Secretion by Stimulated-Microbiota-Specific Lamina Propria T Cells

To further characterize the cellular response of these cells *in vitro*, we evaluated the secretion of pro-inflammatory cytokines by activated LPTC when co-incubated with *L. kefiri*. The stimulation of LPTC with anti-CD3/CD28 induced the secretion of TNF-α, IFN-γ, IL-6 and IL-13. The co-incubation of activated LPTC with the probiotic significantly diminished the secretion of these cytokines (**Figure 3A**, $p < 0.01$, $p < 0.0001$, $p < 0.0001$ and $p < 0.05$ respectively). Also, the incubation of the stimulated cells with CM significantly reduced the levels of the pro-inflammatory cytokines IFN-γ ($p < 0.0001$), IL-6 ($p < 0.001$) and IL-13 ($p < 0.01$).

We then investigated the activation of the NF-κB pathway on LPTC from patients with active IBD in the different culture conditions. We found that p65 levels were diminished in activated cells after a 30 min exposure to *L. kefiri* (**Figure 3B**, $p < 0.01$). *L. kefiri per se* did not trigger the NF-κB pathway.

Next, we analyzed the secretion of the tolerogenic cytokine IL-10 and we found it significantly increased in the supernatant of stimulated LPTC that were exposed to *L. kefiri* (**Figure 3C**, $p < 0.01$). To get a further insight into this suppressive effect, FOXP3 expression was also evaluated in LPTC by flow cytometry (**Figure 3D**). We found that this transcription factor was specifically induced in $CD4^+$ LPTC by *L. kefiri*, irrespective of their activation with anti-CD3/anti-CD28 ($p < 0.05$). We also found that CM induced FOXP3 expression in activated LPTC ($p < 0.01$) (**Figure 3D**).

DISCUSSION

In IBD, dysregulated immune responses take place against the intestinal microbiota in genetically predisposed hosts, and several T cell subsets have been described to be involved in homeostasis breakdown. In this context, microbiota-specific T cells were identified in IBD patients and in animal models (Hepworth et al., 2015; Sorini et al., 2018). Intestinal T cells are target of several therapeutic procedures to constrain inflammation.

In our study, we aimed to evaluate the immunomodulatory properties of a probiotic *Lactobacillus* strain isolated from kefir on lamina propria $CD4^+$ T cells from patients with active IBD. Microbiota-specific lamina propria T cell lines were obtained from intestinal specimens and exposed to *L. kefiri* CIDCA 8348 or conditioned medium to mitigate cell activation. The control of cell proliferation and cytokine secretion of activated T cells correlated significantly with previous results from our group found in healthy mice and in mice fed with a fructose-rich diet that were orally administered with this bacteria (P. Carasi et al., 2015; Zubiría et al., 2017).

Probiotic microorganisms isolated from kefir as well as some of their metabolites have been reported to reduce gut inflammation in colitis animal models, and the amelioration of symptoms after kefir consumption has been observed in one controlled trial with IBD patients to date (Iraporda et al., 2016; Sevencan et al., 2019; Yılmaz, Dolar, and Özpınar 2019). However, this is the first report to show the immunomodulation promoted by *Lactobacillus kefiri* in human organ culture *ex vivo*. The patients in our study were on immunosuppressive and/or immunomodulatory pharmacological treatment, and we considered this could be interfering with our *ex vivo* model results. However, mucosal biopsies obtained from inflamed colon areas of IBD patients showed increased basal levels of pro-inflammatory cytokines IL-6, IL-8 and even IL-1β and IL-17A, reflecting the periods of flares and increased disease activity occurring also in treated patients. We showed that *L. kefiri* CIDCA 8348 suppressed the secretion of these pro-inflammatory cytokines from IBD mucosal biopsies to similar levels as those found in healthy mucosa. Similar results were recently reported in a study performed by Pagnini et al., with a dose-response reduction of TNF-α and IL-17 expression in UC mucosal samples incubated with *L. rhamnosus* GG (Pagnini et al., 2018). In another study, IBD biopsies exposed to the probiotic *Lactococcus lactis* exhibited a reduced secretion of TNF-α and IL-23 (Simčič et al., 2019).

Considering that the gut barrier is impaired in IBD and luminal microorganisms may be found in the lamina propria of patients, we further addressed the effect of *L. kefiri* on lamina propria activated microbiota-specific T cells, to deeper understand whether *L. kefiri*'s anti-inflammatory effect occurred through lamina propria T lymphocyte modulation (Kumar et al., 2020; Al-Sadi et al., 2021). We isolated lamina propria mononuclear cells and established entero-adhesive *E. coli*-specific T cell lines for *in vitro* characterization. LPTC were activated with anti-CD3 and anti-CD28 antibodies, and we found that cell proliferation, cytokine secretion and NF-κB pathway activation were suppressed upon exposure to *L. kefiri*. Collectively, different strains of *Lactobacillus* and *Lactococcus* have shown to modulate the inflammatory activity through multiple mechanisms, but especially by inhibiting the translocation of the nuclear transcription factor NF-κB (Vincenzi et al.,. 2020; Zeng et al., 2020). Here, we co-incubated activated LPTC *in vitro* with *L. kefiri* and we found that NF-κB p65 was diminished in these cells, which also secreted lower amounts of TNF-α, IFN-γ, IL-6 and IL-13 than controls. In addition, activated LPTC incubated with *L. kefiri* CM also exhibited a reduced secretion of these cytokines. However, as proliferation of $CD4^+$ T cells from these LPTC cultures was not diminished by *L. kefiri* CM, we propose that *L. kefiri* effect might be due to a direct interaction between the bacteria and the $CD4^+$ T lymphocytes. To this regard, it has been previously described that the purified S-layer glycoprotein of *L. kefiri* CIDCA 8348 (a regular protein array that completely covers the bacterial surface) could enhance the activation of murine macrophages and bone marrow derived dendritic cells, through the recognition of the S-layer protein glycans by the C-type lectin receptor Mincle (Malamud et al., 2019). However, further investigations are needed to understand the role of the S-layer protein recognition on tuning the activation of antigen-presenting cells triggered by the whole bacteria. Nonetheless, a recent report by Gong et al. showed increased Mincle signaling in both intestinal samples from CD patients and experimental models of colitis, mainly due to an up-regulation of pyroptosis in macrophages, which promotes gut inflammation (Gong et al., 2020). However, the expression of Mincle on human $CD4^+$ T lymphocytes has not been widely reported (Vijayan et al., 2010). Therefore the level of expression in LPTC from IBD patients

and the possible role of this C-type lectin receptor in the interaction between LPTC $CD4^+$ and *L. kefiri* CIDCA 8348 will be key issues to be addressed in further studies. The effect of conditioned medium on cytokine secretion may be attributed to metabolites secreted by lactobacilli. Of note, we have measured lactate present in *L. kefiri* conditioned medium used for the assay and it was 140 ± 0.2 μM. We have performed proliferation and cytokine assays including lactate as a possible modulator (Garrote et al., 2015; Iraporda et al., 2016), but results have been significant for concentrations above 10 mM (data not shown), a value much higher than that secreted by cultured *L. kefiri*. Hence the effect shown in this work cannot be attributed solely to this SCFA and must be further investigated.

Remarkably, in our study we found that LPTC incubated with *L. kefiri* or conditioned medium showed a high frequency of $CD4^+$ $FOXP3^+$ T cells. Several studies highlight that *Lactobacillus* may promote Treg differentiation in animal models (Zakostelska et al., 2011; Smelt et al., 2013; Park et al., 2017). Long term Treg cultures, like the ones performed in this work, have been shown to lose FOXP3 signaling upon repeated stimulation cycles (Hoffmann et al., 2009). In mice, *in vitro* incubation of $CD4^+$ T cells with sonicated extracts of *Lactobacillus rhamnosus* GG diminished IL-17 secretion by these cells and increased FOXP3 and IL-10 secretion via TLR2 mechanism, clearly showing the plasticity induced by the probiotic (Jia et al., 2020). Our results could be showing the functional plasticity of human lamina propria effector T cells in response to the probiotic or its metabolites, which may induce FOXP3 induction. Our experiments showed a direct effect of *L. kefiri* on T cell IL-10 secretion, since no antigen presenting cells were included in our *in vitro* assays. Further studies are needed to characterize whether the $FOXP3^+$ cells are responsible for the increase in IL-10 secretion, and functional assays should be performed to demonstrate the regulatory capacity of these $CD4^+$ $FOXP3^+$ T cells in the future. It is worth noting that the increase of IL-10 secretion induced by the probiotic may be important for generating a tolerogenic milieu in the inflamed gut, even in the absence of FOXP3 induction (Hoffmann et al., 2009). These effects, combined with a lower proliferation of effector $CD4^+$ T lymphocytes and the decrease in pro-inflammatory cytokine secretion could be a useful complement of the adequate drug treatment to shift the IBD gut toward a tolerogenic state.

Although the anti-inflammatory activity of *L. kefiri* CIDCA 8348 has been shown in mice (Carasi et al., 2015; Zubiría et al., 2017), this is the first report demonstrating the immunomodulatory properties of a kefir-isolated strain (*L. kefiri* CIDCA 8348) on human intestinal tissue and primary T cells from IBD patients. Great effort has been made to modulate the pro-inflammatory activity of T cells as a treatment for IBD, with variable success; however, there is still need for improving therapies. Understanding the mechanisms of interaction between probiotics and immune cells in the gut could open new avenues to help prevent or treat inflammatory bowel disease.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité de Ética e Investigaciones, Hospital de Gastroenterología Dr. Carlos Bonorino Udaondo Av Caseros 2,153, 3er Piso 1,264 Ciudad de Buenos Aires. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors have made substantial contributions to the study. Conception and design of the study: CM and MS. Acquisition of data: RC, CM, KC, IS, EBR. Intestinal biopsies or surgical pieces sampling: AR, ID, EP, SB, GC, MY and LG. Patients recruitment and clinical diagnosis: AS, SG, PT, GC and MY. Analysis and/or interpretation of data: CM, MS, GD, RC and KC. Drafting the manuscript: CM, MS, RC. Revising the manuscript critically for important intellectual content: CM, MS, GD, RC, KC, and AS. Approval of the version of the manuscript to be published: CM, MS, GD, RC, KC, IS, EBR, AR, ID, EP, SB, GC, MY, LG, AS.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.658026/full#supplementary-material.

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Psychoactive Effects of *Lactobacillus johnsonii* Against Restraint Stress-Induced Memory Dysfunction in Mice Through Modulating Intestinal Inflammation and permeability—a Study Based on the Gut–Brain Axis Hypothesis

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Though the underlying mechanism remains elusive, a close relationship between psychological stress and intestinal inflammation has been widely accepted. Such a link is very important to set the basis for our understanding of the critical role of gut-brain axis (GBA) in homeostatic processes in health and disease. Probiotics that could confer benefits to mental health through GBA are referred to as "psychobiotics". This study aimed to further determine whether a potential psychobiotic strain, *Lactobacillus johnsonii* BS15 could prevent memory dysfunction in mice induced by psychological stress through modulating the gut environment, including intestinal inflammation and permeability. Memory dysfunction in mice was induced by restraint stress (RS), one of the most commonly utilized models to mimic psychological stress. The mice were randomly categorized into three groups including no stress (NS), restraint stress (RS), and probiotic (RS-P) and administered with either phosphate buffered saline (NS and RS groups) or *L. johnsonii* BS15 (RS-P group) every day from day 1–28. From days 22–28, the mice in RS and RS-P groups were subjected to RS each day. Results revealed that BS15-pretreatment enhanced the performance of RS-induced mice during three different behavioral tests for memory ability and positively modulated the hypothalamic–pituitary–adrenal axis by attenuating the serum corticosterone level. In the hippocampus, *L. johnsonii* BS15 positively modulated the memory-related functional proteins related to synaptic plasticity, increased neurotransmitter levels, and prevented RS-induced oxidative stress and mitochondria-mediated apoptosis. In the intestines, *L. johnsonii* BS15 protected the RS-induced mice from damaged gut barrier by enhancing the mRNA levels of tight junction proteins and exerted beneficial effects on the anti-inflammatory cytokine levels reduced by RS. These findings provided more evidence to reveal the psychoactive effect of *L. johnsonii* BS15

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against memory dysfunction in RS-induced mice by modulating intestinal inflammation and permeability.

Keywords: *Lactobacillus johnsonii*, psychobiotics, gut-brain axis, memory dysfunction, intestinal environment

INTRODUCTION

When provided in adequate amounts, probiotics could exert beneficial effects on the host (Sherman et al., 2009), such as improving the intestinal barrier function and gut microbiota, reducing proinflammatory cytokines, and increasing the intestinal antioxidant ability, and have been utilized for the prevention and/or treatment of many different intestinal diseases, such as inflammatory bowel disease (Wasilewski et al., 2015) and diarrhea (Selinger et al., 2013). However, the exact mechanism underlying these effects has not been identified (Guandalini et al., 2015; Sun et al., 2016). For example, Je et al., 2018 proved that ID-JPL934, a mixture of three probiotic strains (two *Lactobacillus* strains and one *Bifidobacterium* strain at a 1:1:1 ratio) attenuates dextran sulfate sodium-induced colitis by inhibiting the mRNA expression levels of proinflammatory cytokines in rodents. Probiotics also prevent or treat metabolic disorders and many other diseases (Le Barz et al., 2019). Given the close relationships between intestinal tracts and other organs, researchers have focused on expanding the application scope of probiotics.

Gut–brain axis (GBA) is defined as a network and communication among gastrointestinal tract, the enteric nervous system, and the brain. (Sudo et al., 2004) observed substantially high serum corticosterone level and reduced mRNA expression levels of brain-derived neurotrophic factor (BDNF) in the hippocampus and cortex in response to restraint stress (RS) in germ-free mice (born and fed entirely in the absence of microorganisms), indicating that commensal microbiota in the intestines could affect post-natal development. Effects on cognitive abilities including poor learning and memory and autism-like behavior were also found (Desbonnet et al., 2015; Vuong and Hsiao, 2017). The application of probiotic or prebiotic could lead to enhanced long-term potentiation (an experimentally evoked process in which the synaptic strength is rapidly increased and involves the crucial mechanism underlying learning and memory), increased BDNF concentrations, and improved intestinal immunity and barrier function, which consequently enhance the performance on a number of learning and memory tests (Zareie et al., 2006; Dash et al., 2015; Vazquez et al., 2015).

The association between intestinal environment and host behavior and the potential psychobiotics/probiotics that benefit mental health and yield positive psychiatric effects in psychopathology through GBA have been widely researched (Sarkar et al., 2018). Sgritta and colleagues (2019) reported consistent and robust reversal for social behavioral deficits by a potential psychobiotic, *Lactobacillus reuteri* in four different autistic spectrum disorder (ASD) mouse models (*Shank3B*$^{-/-}$ mice, valproic acid-treated mice, BTBR mice and germ-free mice). Lee et al., 2018 also found that *Lactobacillus plantarum* C29 could alleviate memory impairment in 5XFAD transgenic mice, indicating its possible ability to prevent Alzheimer's disease. However, the limitation of psychobiotic researches should not be ignored that most reported findings about their effects opertains to rodent models rather than human studies. Also, most psychobiotic research findings are currently understood in terms of correlation rather than causation. Therefore, in order to make a potential psychobiotic strain convinceing enough to be applied in human studies, the mechanism underlying the beneficial effect must be considered to provide information for psychobiotic exploration based on GBA.

Lactobacillus johnsonii BS15 (CCTCC M2013663) was isolated from homemade yogurt from Hongyuan Prairie, Aba Autonomous Prefecture, China and was found to prevent non-alcoholic fatty liver disease by attenuating hepatic inflammation and mitochondrial injury and improving gut environment in obese mice (Xin et al., 2014). It also effectively prevents memory dysfunction induced by chronic high-fluorine intake by modulating the intestinal environment (Sun et al., 2020). Recently, we also found that *L. johnsonii* BS15 pretreatment enhanced intestinal health and prevented the hippocampus-related memory dysfunction induced by water avoidance stress (WAS), a well-established model for causing psychological stress (Wang et al., 2020). However, more evidence needs to be provided to prove whether or not *L. johnsonii* BS15 could be applied as a qualified psychobiotic that positively influences and protects mental health and cognitive behaviors against psychological stressors.

This study aimed to determine whether *L. johnsonii* BS15 could effectively prevent memory dysfunction in mice after restraint stress (RS) through modulating the gut environment, including intestinal inflammation and permeability. RS was induced in C57BL/6J mice to determine whether *L. johnsonii* BS15 could prevent memory dysfunction by conducting different behavioral tests. Given that the hippocampus is considered as a crucial brain region in memory ability and a neurobiological mediator underlying the bacteria-cognition link (Stachenfeld and Botvinick, 2017), the levels of memory-related functional proteins and neurotransmitters, antioxidant capacity, and apoptosis level were measured to reveal how *L. johnsonii* BS15 rescuing the impaired memory ability under RS influences the hippocampus. Intestinal integrity and inflammatory factors were also evaluated to further understand the mechanism on how *L. johnsonii* BS15 prevents hippocampus-related memory dysfunction.

MATERIALS AND METHODS

Bacteria Preparation and Animal Treatment

L. johnsonii BS15 was maintained in de Man, Rogosa and Sharpe (MRS, QDRS Biotec, Qingdao, Shandong, China) broth under

anaerobic environment at 37 C for 36 h. Heterotrophic plate count was used to evaluate the amount of bacterial cells. After collection, the bacterial cells were washed with saline and then suspended at pH 7.0 in phosphate buffered saline (PBS) at a concentration of 1×10^9 cfu *L johnsonii* BS15/mL. Our previous experiment confirmed that oral gavage of *L. johnsonii* BS15 at the daily amount of 0.2 ml solution with 1×10^9 cfu *L johnsonii* BS15/mL has the best preventive effects for obese mice (Xin et al., 2014).

A total of 108 5C7BL/6J male mice (3 week-old) were provided by Dashuo Biological Institute (Chengdu, Sichuan, China). The animals were fed on normal chow diet for 1 week before treatment to stabilize all metabolic conditions. All mice were housed with a 12 h light/dark cycle (lights on at 8:00 a.m. and off at 8:00 p.m.) in a room with strictly controlled temperature of 20–22 C and humidity of 40–60%. The animals were randomly divided into three groups each containing six cages (six mice per cage) and administered with either PBS (pH 7.0) (NS and RS groups) or *L. johnsonii* BS15 (RS-P group; daily amounts of 2×10^8 cfu) through oral gavage from day 1–28. All animal experiments followed the guidelines for the use and care of laboratory animals (approval number: SYXKchuan 2019–187; approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University).

Study Design and Sampling

The first day after 1 week stabilization was defined as day 1. From day 22–28, the mice in RS and RS-P groups were subjected to RS by placing them in 50 ml plastic conical centrifuge tubes for 60 min each day and directing their head toward the nasal end of the cylinder with air vents. The tubes restrained all physical movements without subjecting the animal to pain. All mice were not provided with food and water during the RS experiment.

After RS experiment in RS and RS-P groups on the morning of day 28, 8–10 mice from the three experimental groups were randomly selected and immediately sacrificed through cervical dislocation in accordance with institutional guidelines of animal care. Blood was collected through cardiac puncture, and the samples were immediately placed on ice and centrifuged. The isolated serum was frozen at −20 C until further analysis. The hippocampus and epithelial tissues of the jejunal and ileac samples were immediately removed from mice. After being washed by ice-cold sterilized saline, these samples were frozen in liquid nitrogen and then stored at −80 C. The other parts of hippocampus, the mucosa of jejunum and ileum were separately removed and frozen at −20 C until further analysis. The samples stored at −80 C were retrieved, and RNA of hippocampus, jejunum, and ileum was extracted using E. Z.N.A. Total RNA Kit (OMEGA Bio-Tek, Doraville, GA, United States) in accordance with the manufacturer's guidelines. Total RNA (1 μg) was synthesized into first-strand complementary DNA (cDNA) using PrimeScriptTM RT reagent kit with gDNA Eraser (TaKaRa, Dalian, Liaoning, China). The cDNA products were stored at −20 C until subsequent tests. Another four mice in each group were sacrificed and their brain, jejunum and ileum was removed, fixed in 4% paraformaldehyde solution, and stored in 4 C for immunohistochemical and/or immunofluorescent assay.

In a subset of experiments, 10–12 mice from each group were selected for T-maze test. The habituation and training phases of T-maze test were performed at day 21 and lasted for 6 days until the 27th experimental day. On day 28, after the RS treatment for RS and RS-P groups, the testing phase of T-maze test was conducted for all three experimental groups. Another 10–12 mice from each group were selected for novel object and passive avoidance tests. Novel object test was carried out on day 28 after the mice were subjected to RS, except mice in NS group. The mice were allowed to have a 10 min rest period between two tests and then placed in the wooden box for passive avoidance test to experience familiarization and training. The testing phase for passive avoidance test was conducted on day 29. All the mice that underwent behavioral tests were not selected to avoid the carry-over effects of behavioral testing on inflammation related parameters and other biomarkers (Boitard et al., 2014; Beilharz et al., 2017). **Figure 1** displays the flow diagram of behavioral tests applied in this study.

Behavioral Tests

Novel Object Test

Mice have the tendency to investigate a novel object rather than a familiar one. On this basis, novel object test was undertaken for hippocampus-dependent memory formation following the method described by Gareau et al., 2011 with minor modification. The test was briefly described below:

The mice without RS were placed into a dark open-field arena (40 × 40 × 45 cm, l×b×h) and allowed to freely explore for 1 h for habituation. The following two different objects were exposed to the mice after RS or habituation: blue and orange tube caps with the same size and shape and a smooth pebble of proper size. Behavioral assessment consisted of the following two phases:

Familiarization Phase

The mice were placed into the arena with a blue tube cap and an orange tube cap placed in opposite corners and allowed to freely explore the two objects for 5 min. The objects were then removed, and the mice were given a rest period (20 min) before the testing phase.

Testing Phase

The orange tube cap was replaced by the smooth pebble during the rest period, and the mice after resting were re-exposed to the blue tube cap and the smooth pebble. Memory could be evaluated as the frequency to explore the smooth pebble. compared with the blue tube cap during the testing phase. Exploration ratio represents the smelling bouts proportion related to the new object vs. the old one (ratio of the frequency of smelling the smooth pebble to the total frequency of smelling the blue tube cap and the smooth pebble). A ratio of 0.5 represents impaired hippocampus-dependent memory as no discrimination is found between the two objects. In the present test, exploration was defined by

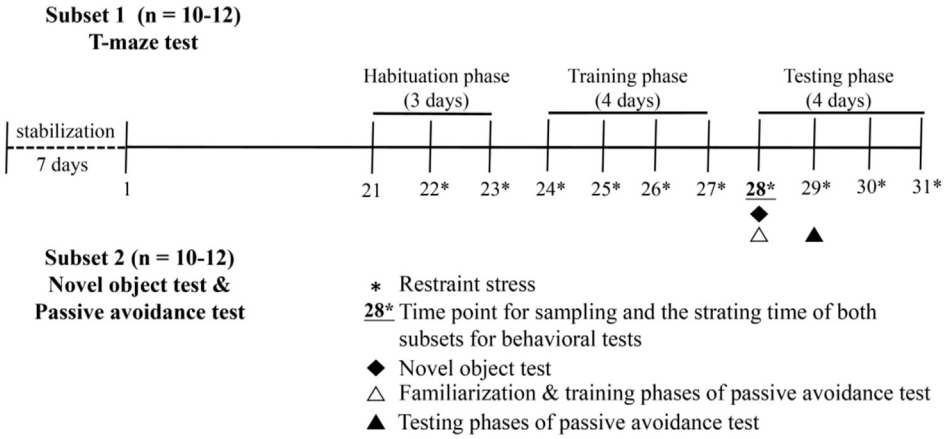

FIGURE 1 | Flow diagram of behavioral tests.

orientating the mice toward the object with the nose pointing directly to the object within 1–2 cm.

Passive Avoidance Test

Memory acquisition and retention were evaluated using step-down passive avoidance test. The apparatus was a wooden box (40 × 40 × 40 cm, l×b×h) with floor consisted of 0.3 cm caliber stainless paralleled steel bars. The bars were spaced 1 cm apart. A small platform (4 × 4 × 4 cm, l×b×h) was placed in the center of the grid floor.

Familiarization and Training Phases

For familiarization, the mice placed on the platform in the wooden box were allowed to freely explore for 3 min. The training phase was then started after the mice became familiar with the apparatus. In the training phase, the mice were gently placed on the platform (3 min) and immediately received 2 s of electric shock (36 V, 1 mA, 50 Hz) once they stepped down with all four paws on the grid. Afterward, the animals generally returned to the platform.

Testing Phase

The testing phase (3 min) was started 24 h after the training phase with identical process, and RS was induced in advance when needed. Escape latency was recorded as the duration the mice first stayed on the platform. Error number was measured as the repeated times the mice stepped down on the grid during the phase (3 min) (Malekmohamadi et al., 2007). Poor memory ability could be indicated by short escape latency and high error numbers (Chen et al., 2014).

T-Maze Test

The applied T-maze was a "T"-shape enclosed apparatus with a start arm (60 × 10 × 20 cm, l×b×*h*) and two goal arms (30 × 10 × 20 cm, l×b×h). Given their natural tendency to explore a novel environment, the mice were first placed at the base of the start arm and allowed to freely enter one of the goal arms. A mouse tends to choose the other goal arm which is not visited prior to the second trial, and this phenomenon could reflect the memory of the first choice. Alternation is sensitive to memory dysfunction (especially hippocampus-related) and represents a model of working memory (Albert-Gasco et al., 2017).

Rewarded T-maze test was chosen (over spontaneous T-maze test) because mice could run many trials per day before getting sated (Deacon and Rawlins, 2006). As food reward, 0.07 ml of 1:1 (vol/vol) full fat/water sweetened condensed milk (Nestle, Qingdao, Shandong, China) mixture was given per trial by preset pipette. The test was briefly described below:

Habituation and Training Phases

During habituation phase (3 days), the mice were softly stroked, slowly picked up, and put down three times per day (3 min each time) to ensure that they were accustomed to the touch from operators. Given that they are wary of eating anything new (Forestier et al., 2018), the mice were fed 0.5 ml of food reward each day to get familiar with its taste. Training phase (4 days) was performed after habituation, and the animals were placed into T-maze with all arms open and allowed to explore freely for 10 min. The mice in the start arm were allowed to run toward one goal arm, and their reward was provided into the food well while the other arm was blocked by its door. No more than 3 min of training time was given until the mice discovered that the well was empty. Each mouse was trained four times per day (left and right runs were given with equal numbers).

Testing Phase

Each mouse was tested for 4 days with five trials per day. The rodent was allowed to explore the whole maze without loss of interest. RS was given before the test started each day when needed. With one of the goal arms blocked (randomly chosen for each trial), the mice from the start arm were allowed to run toward the open goal arm with consumable reward. They were immediately returned to the start arm as soon as they found the well empty, and the operator opened the door of the blocked goal arm. With 0 s (for trails at day 1 and 2) or 1 min (for trails at day 3 and 4) of retention interval, the mice were allowed to run from the

start arm again and choose one arm. If it chose the correct arm, then the mouse was allowed to consume the reward; if incorrect, the mouse was removed after definitively discovering that the well was empty. Working memory with 0 s and 1 min of retention interval was separately assessed as a ratio of correct times to total trail times (n = 10).

Biochemical Analysis

Corticosterone and D-lactate serum contents and diamine oxidase (DAO) activity were quantified using ELISA) kits specific for mice (MLBIO Biotechnology Co., Ltd, Shanghai, China) following the manufacturer's instructions. The standard curve was used to calculate the contents of determined proteins. In the hippocampus, the contents of neurotransmitters (dopamine, DA; 5-hydroxytryptamine, 5-HT; acetylcholine, Ach; glutamic acid, Glu; gamma-aminobutyric acid, GABA; nitric oxide, NO), neurotransmitter-related proteins (including nitric oxide synthase, NOS; acetyl-cholinesterase, AchE; choline acetyltransferase, ChAT), and two apoptosis-related proteins (Bax and Bcl-2) were determined by ELISA using reagent kits specific for mice (MLBIO Biotechnology Co., Ltd, Shanghai, China) in accordance with the manufacturer's instructions. The antioxidant indexes in the hippocampus were measured using commercial kits (Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China), including the activities of catalase (CAT), total antioxidation capacity (T-AOC), glutathione peroxidase (GSH-Px), superoxide dismutase, and malondialdehyde (MDA) and GSH contents (Zareie et al., 2006). Inflammatory factors contents in the jejunum and ileum were also determined by ELISA using reagent kits specific for mice (MLBIO Biotechnology Co., Ltd, Shanghai, China). The determined inflammatory factors included interleukin (IL)-1β, IL-4, IL-6, IL-10, tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ).

Immunohistochemistry

Tissues for immunohistochemical assay were embedded by paraffin and cut by a microtome. Using a microwave oven (model: P70D20TL-P4; Galanz, Guangdong, China), slices were submerged in citrate antigen retrieval solution and heated at medium heat until boiling. The temperature was then ceased and tissues were kept warm for 8 min. The tissues were heated at medium–low heat for 7 min. The slices after free cooling were placed into PBS (pH 7.4) and shaken for 5 min for decoloration, which was repeated three times. The sections were then incubated in 3% oxydol for 25 min at room temperature and away from the light to block endogenous peroxidase. The slices were washed three times in PBS by shaking for 5 min, then sealed for 30 min by 3% bull serum albumin, and incubated with monoclonal rabbit anti-BDNF (1:400) or polyclonal rabbit anti-CREB (1:500) antibodies at 4°C overnight. Species-specific biotinylated anti-rabbit immunoglobulin (horseradish peroxidase labeled) was used for immuno-detection. Following the second antibody incubation, the 3,3′-diaminobenzidine staining kit was used to complete the reaction according to the manufacturer's instructions. Hematoxylin staining was performed to re-stain the nucleus.

Immunofluorescence

The 5-μm-thick paraffin-embedded jejunal and ileal tissue sections were used for immunofluorescence. Heat-induced antigen retrieval was performed by autoclaving the sections for 10 minutes at 121°C in 10mM sodium citrate buffer (pH 6.0). The sections were blocked with 8% skim milk in TBST at 37°C for 40 minutes, and then immunostained using primary antibodies against ZO-1 (1:200, GB11195, rabbit; Servicebio), occludin (1: 200, GB111401, rabbit; Servicebio), claudin-1 (1:200, GB11032, rabbit; Servicebio) at 4°C overnight. The sections were washed and incubated with secondary fluorescent antibodies at 37°C for 60 minutes. The secondary antibodies was CY3 goat anti-rabbit IgG (1:300; GB21303; Servicebio). Sections were mounted with Nikon DS-U3 with DAPI (G1012, Servicebio). Images were captured with an Nikon Eclipse C1 fluorescence microscope (Nikon, Tokyo, Japan).

Real-Time Quantitative Polymerase Chain Reaction (qPCR) Analysis

PCR was performed to determine the prepared cDNA products from the hippocampus, jejunum, and ileum. A CFX96 Real-time PCR Detection System (Bio-Rad, Hercules, CA, United States) with iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, United States) was used with the following protocol: 5 min at 95 C, 40 cycles of 10 s denaturation at 95 C, and 30 s annealing/extension at optimum temperature (**Tables 1**, **2**). PCR product purity was monitored by a final melting curve analysis. Standard curves were obtained through serial dilution. The primer sequences for targeted genes are presented in **Table 1**. $_{\Delta\Delta}$Ct method was applied to estimate mRNA abundance. The samples (n = 6) in each group were analyzed in triplicate, and Ct was calculated as $(Ct_{target} - Ct_{\beta\text{-actin}})_{treatment} - (Ct_{target} - Ct_{\beta\text{-actin}})_{control.}$ β-actin was used as the eukaryotic housekeeping gene to normalize relative gene expression levels. Mean values of measurements were applied to evaluate the mRNA expression levels of cyclic amp (cAMP) response element binding protein (CREB), N-methyl-D-aspartate receptor (NMDAR), brain-derived neurotrophic factor (BDNF), c-Fos, stem cell factor (SCF), neural cell adhesion molecule (NCAM), Bcl-2, Bad, Bcl-xL, Bax, caspase-3 and caspase-9 in the hippocampus and IL-1β, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IL-4, IL-10, IL-6, claudin-1, occludin, and zoluna occludens protein (ZO)-1 in the jejunum and ileum.

qPCR Quantification

The population of total bacteria and *Lactobacillus johnsonii* was estimated in the jejunum and ileum following the method of Xin and colleagues (2014). A CFX Connect™ real-time system (Bio-Rad, Hercules, CA, United States) and SYBR® Premix Ex Taq™ II (TaKaRa, Dalian, Liaoning, China) were used to perform qPCR. **Table 3** presents the primers for the qPCR of the microbiota. The

TABLE 1 | Primer sequences for RT-qPCR in hippocampus.

Gene	Tm (°C)	Sequence	References
β-actin	60	F: GCTCTTTTCCAGCCTTCCTT R: GATGTCAACGTCACACTT	Sun et al. (2020)
BDNF	60	F:GCGCCCATGAAAGAAGTAAA R: TCGTCAGACCTCTCGAACCT	Niu et al. (2018)
c-Fos[a]	59.5	F:CAGAGCGGGAATGGTGAAGA R:CTGTCTCCGCTTGGAGTGTA	—
NCAM	60	F: GGGAACTCCATCAAGGTGAA R: TTGAGCATGACGTGGACACT	Niu et al. (2018)
SCF	60	F:CCTTATGAAGAAGACACAAACTTGG R:CCATCCCGGCGACATAGTTGAGGG	Niu et al. (2018)
CREB	60	F: CCAGTTGCAAACATCAGTGG R: TTGTGGGCATGAAGCAGTAG	Niu et al. (2018)
NMDAR	60	F: GTGGATTGGGAGGATAGG R: TTAGTCGGGCTTTGAGG	Niu et al. (2018)
Caspase-9	61	F: GAGGTGAAGAACGACCTGAC R: AGAGGATGACCACCACAAAG	Guo et al. (2017)
Caspase-3	59	F: ACATGGGAGCAAGTCAGTGG R: CGTCCACATCCGTACCAGAG	Guo et al. (2017)
Bax	61	F: ATGCGTCCACCAAGAAGC R: CAGTTGAAGTTGCCATCAGC	Guo et al. (2017)
Bad	60	F: AGAGTATGTTCCAGATCCCAG R: GTCCTCGAAAAGGGCTAAGC	Guo et al. (2017)
Bcl-2	61	F: AGCCTGAGAGCAACCCAAT R: AGCGACGAGAGAAGTCATCC	Guo et al. (2017)
Bcl-xl	62	F: TGTGGATCTCTACGGGAACA R: AAGAGTGAGCCCAGCAGAAC	Guo et al. (2017)

[a]*The primer sequences of c-fos is designed by National Center for Biotechnology Information (NCBI) and the referenced gene ID is 14,281.*

TABLE 2 | Primer sequences for RT-qPCR in small intestines.

Gene	Tm (°C)	Sequence	References
β-actin	60	F: GCTCTTTTCCAGCCTTCCTT R: GATGTCAACGTCACACTT	Sun et al. (2020)
Claudin-1	60	F:GGGGACAACATCGTGACCG R:AGGAGTCGAAGACTTTGCACT	Liu et al. (2017)
Occludin	60	F:TTGAAAGTCCACCTCCTTACAGA R:CCGGATAAAAAGAGTACGCTGG	Liu et al. (2017)
ZO-1	60	F:GATCCCTGTAAGTCACCCAGA R:CTCCCTGCTTGCACTCCTATC	Liu et al. (2017)
TNF-α	59.0	F:ACGGCATGGATCTCAAAGAC R:AGATAGCAAATCGGCTGACG	Xin et al. (2014)
IL-1β	60	F:ATGAAAGACGGCACACCCAC R:GCTTGTGCTCTGCTTGTGAG	Liu et al. (2017)
IL-6	60	F:TGCAAGAGACTTCCATCCAGT R:GTGAAGTAGGGAAGGCCG	Liu et al. (2017)
IFN-γ	53	F:TCAAGTGGCATAGATGTGGAAGAA R:TGGCTCTGCAGGATTTTCATG	Liu et al. (2017)
IL-10	56	F:GGTTGCCAAGCCTTATCGGA R:ACCTGCTCCACTGCCTTGCT	Liu et al. (2017)
IL-4	55	F:ACAGGAGAAGGGACGCCAT R:GAAGCCCTACAGACGAGCTCA	Usuda et al. (2012)

reaction mixture (25 μL) included SYBR® Premix Ex TaqTM II (12.5 μL), forward and reverse primers (1 μL), sterile deionized water (9.5 μL), and DNA template (1 μL). PCR was performed as follows: 95 C for 1 min, 40 cycles of 94 C for 15 min, and annealing at optimal temperatures for 30 s at 72 C. The specificity of the PCR primers was regulated by generating melting curves.

Data Analysis

Data were analyzed based on individual mice. Statistical analysis was performed using one-way ANOVA, followed by Duncan's multiple-range test for multiple comparisons (both normality test and equal variance test passed) (SigmaPlot for Social Sciences version 12). Differences at $P < 0.05$ were considered statistically significant.

TABLE 3 | Primer information on the microflora for qPCR.

Target species	Tm (°C)	Primer sequence (5→3)	References
Total bacteria	60.0	F: CGGYCCAGACTCCTACGGG R: TTACCGCGGCTGCTGGCAC	Xin et al. (2014)
L. johnsonii	61.4	F:CACTAGACGCATGTCTAGAG R:AGTCTCTCAACTCGGCTATG	Xin et al. (2014)

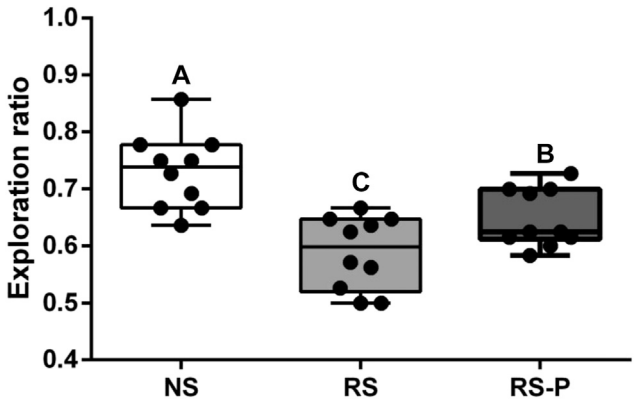

FIGURE 2 | Effects of *L. johnsonii* BS15 on exploration ratio by novel object test. Data are presented with the means ± standard deviation (n = 10). Bars with different letters are significantly different on the basis of Duncan's multiple range test ($P < 0.05$).

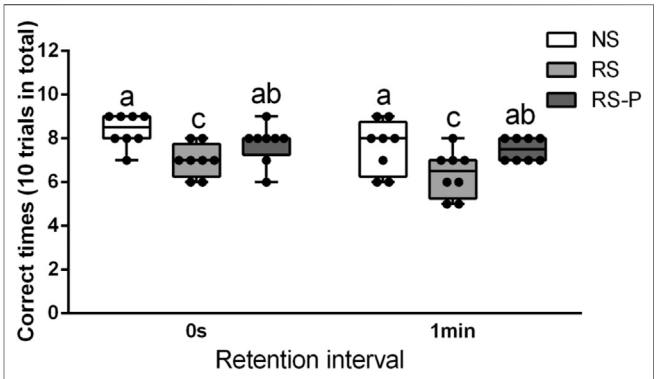

FIGURE 4 | Effects of *L. johnsonii* BS15 on the correct times with both 0s and 1min of retention interval by T-maze test. Data are presented with the means ± standard deviation (n = 8). Bars with different letters are significantly different on the basis of Duncan's multiple range test ($P < 0.05$).

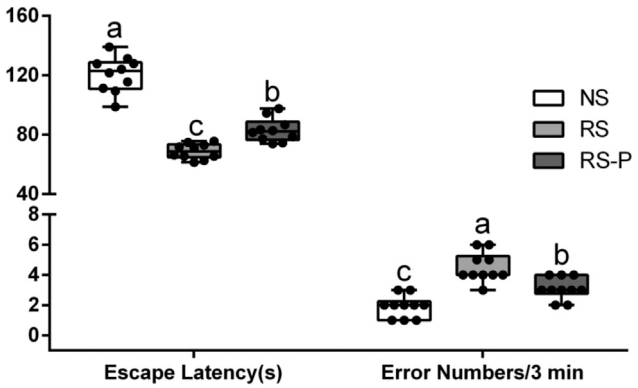

FIGURE 3 | Effects of *L. johnsonii* BS15 on the escape latency and error numbers by passive avoidance test. Data are presented with the means ± standard deviation (n = 10). Bars with different letters are significantly different on the basis of Duncan's multiple range test ($P < 0.05$).

RESULTS

Behavioral Tests

Figures 2–4 show the results of behavioral tests for memory abilities. Significantly lower time in exploration ratio (**Figure 2**) and escape latency (**Figure 3**) were observed ($P < 0.05$) in the RS group compared with those in the NS group. Correct times for 0 s and 1 min of retention interval (**Figure 4**) in the RS group were also significantly lower ($P < 0.05$) than those in the NS group. Meanwhile, error numbers (**Figure 2**) in the RS group were significantly higher than those in the NS group. Positive changes in all indexes were induced by *L. johnsonii* BS15 in the RS and RS-P groups. In particular, the exploration ratio (**Figure 2**), escape latency (**Figure 3**), and correct times for both 0 s and 1 min of retention interval (**Figure 4**) were significantly high ($P < 0.05$) in the RS-P group. The error numbers (**Figure 3**) in the RS group were significantly lower ($P < 0.05$) than those in the RS-P group. Moreover, significant differences ($P < 0.05$) in exploration ratio (**Figure 2**), escape latency, and error numbers (**Figure 3**) were observed between the RS-P and NS groups, whereas the correct times showed no significance ($P > 0.05$) (**Figure 3**).

Serum Corticosterone and Memory-Related Functional Proteins

Figure 5 shows the differences in corticosterone levels in the serum among three groups. Corticosterone level was significantly higher ($P < 0.05$) in the RS group compared with that in the other groups, and that in the RS-P group was significantly higher ($P < 0.05$) than that in the NS group. Levels of memory-related functional proteins are shown in **Figure 6**. All indexes were significantly lower ($P < 0.05$) in the RS group than in the NS group (**Figures 6A–F**). Although no significant change ($P > 0.05$) in NCAM (**Figure 6C**) was observed between the RS and RS-P groups, the mRNA expression levels of BDNF, CREB, SCF, c-Fos, and NMDAR in RS-P group were significantly higher ($P < 0.05$) than those in the RS group (**Figures 6A,B,D–F**). Except for CREB

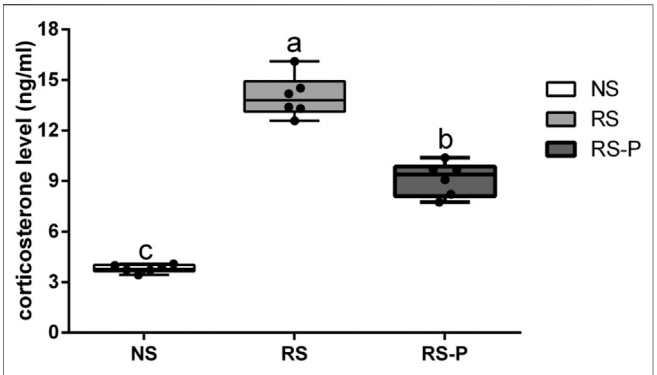

FIGURE 5 | Levels of corticosterone in the serum. Data are presented with the means ± standard deviation (n = 6). Bars with different letters are significantly different on the basis of Duncan's multiple range test ($P < 0.05$).

with no significant difference ($P > 0.05$), all other indexes in the RS-P group were significantly lower ($P < 0.05$) than those in the NS group. As shown in **Figure 6G–L**, the protein expression levels of BDNF and CREB were significantly reduced in the RS group compared with that in the other two groups.

Neurotransmitters and Related Functional Proteins

Figures 7A–C show that although DA, 5-HT, and Ach levels were significantly lower in the RS group than those in the NS group, they were significantly increased ($P < 0.05$) by *L. johnsonii* BS15 in RS-P group and showed no significant differences ($P > 0.05$) compared with those in the NS group. In addition, the Glu content (**Figure 7D**) and AchE activity (**Figure 7G**) in the RS group were significantly higher ($P < 0.05$) than those in the other groups but were influenced by *L. johnsonii* BS15 to show no significant difference ($P > 0.05$) between the NS and RS-P groups. As shown in **Figure 7E**, the GABA content in the RS-P group was significantly higher ($P < 0.05$) than that in the RS group but lower ($P < 0.05$) than that in the NS group. As shown in **Figure 7H**, the ChAT activity in the RS group was significantly decreased ($P < 0.05$) compared with that in the NS group but was not significantly different ($P > 0.05$) in the RS-P group compared with the NS or RS group. The levels of NO and NOS activity (**Figures 7F,I**) were not significantly influenced ($P > 0.05$) by BS15 and RS.

Antioxidant Capacity and Apoptosis

Figure 8 demonstrates antioxidant indexes in the hippocampus. As shown in **Figure 8A**, T-AOC was significantly lower ($P < 0.05$) in the RS and RS-P groups than that in the NS group, but no difference was shown ($P > 0.05$) between the two RS groups. No changes were observed ($P > 0.05$) in SOD activity (**Figure 8B**). **Figures 8C,E** show significantly lower CAT activity ($P < 0.05$) and higher MDA content ($P < 0.05$) in the RS group than in the other two groups, but no significant differences of these two indexes were found ($P > 0.05$) between the NS and RS-P groups. Meanwhile, GSH-Px activity and GSH content were significantly low ($P < 0.05$) in the RS-P group (**Figures 8D,F**) but showed no differences ($P > 0.05$) compared with those in the NS or RS group. The results of apoptosis-related functional protein contents and mRNA expression levels in the hippocampus are presented in **Figure 9**. Significantly lower values of bcl-2 protein content (**Figure 9A**) and mRNA expression levels of bcl-2 (**Figure 9A**) and Bcl-xL (**Figure 9C**) were found ($P < 0.05$) in the RS groups than those in the NS and RS-P groups. Higher protein and mRNA expression levels of Bax (**Figure 9B**) and caspase-3 (**Figure 9F**) were also found ($P < 0.05$) in the RS groups. These indexes showed no significant differences ($P > 0.05$) between the NS and RS-P groups. However, the mRNA expression levels of Bad (**Figure 9D**) and caspase-9 (**Figure 9E**) remained unchanged ($P > 0.05$).

Intestinal Integrity and Permeability

The contents of DAO and D-lactate in the serum are shown in **Figure 10A**. The mRNA expression levels of three tight junction proteins in the jejunum and ileum are presented in **Figures 10B–D** (occludin, claudin-1, and ZO-1). As shown in **Figure 10A**, the DAO level in the RS-P group was significantly higher ($P < 0.05$) than that in the NS group but lower ($P < 0.05$) than that in the RS group. A significantly higher D-lactate level was observed ($P < 0.05$) in the RS group relative to other two groups. The D-lactate levels showed no differences ($P > 0.05$) between NS and RS-P groups. Except for occludin in the jejunum (**Figure 10C**) of which the mRNA expression level was higher ($P < 0.05$) than NS group, all mRNA expression levels in jejunum and ileum in the RS-P group were higher ($P < 0.05$) than those in the RS group, but no significant differences ($P > 0.05$) were found between the NS and RS-P groups (**Figures 10B–D**). The protein expressions of three tight junction proteins were also detected by immunofluorescence, and the results showed the same trend (**Figures 11**, **12**).

Inflammatory Factors

Results of protein contents and mRNA expression levels of inflammatory factors are shown in **Figure 13**. The inflammatory factors showed significant differences ($P < 0.05$, **Figures 13A–F**), although a small proportion of the indexes remained unchanged ($P > 0.05$, **Figures 13C–E**) among the three experimental groups (protein contents of IL-1β, IL-6, and IL-4 and mRNA expression of IL-6). As shown in **Figures 13A,B**, the mRNA expression levels of TNF-α and IFN-γ and the protein contents of IFN-γ were significantly up-regulated ($P < 0.05$) in the RS group compared with those in the other groups. However, the changes were not controlled by *L. johnsonii* BS15 because no significant differences were detected ($P > 0.05$) between the NS and RS-P groups. In addition, the mRNA expression levels of IFN-γ (**Figure 13B**) and IL-1β (**Figure 13C**) in the RS group were significantly higher ($P < 0.05$) than those in the NS group, and these indexes in the RS-P group showed no differences ($P > 0.05$) compared with those in the NS or RS group. Different from the protein content, the mRNA expression level of IL-4 was influenced by *L. johnsonii* BS15 because it was significantly up-regulated ($P < 0.05$) in the RS-P group than that in the NS and RS groups (**Figure 13E**).

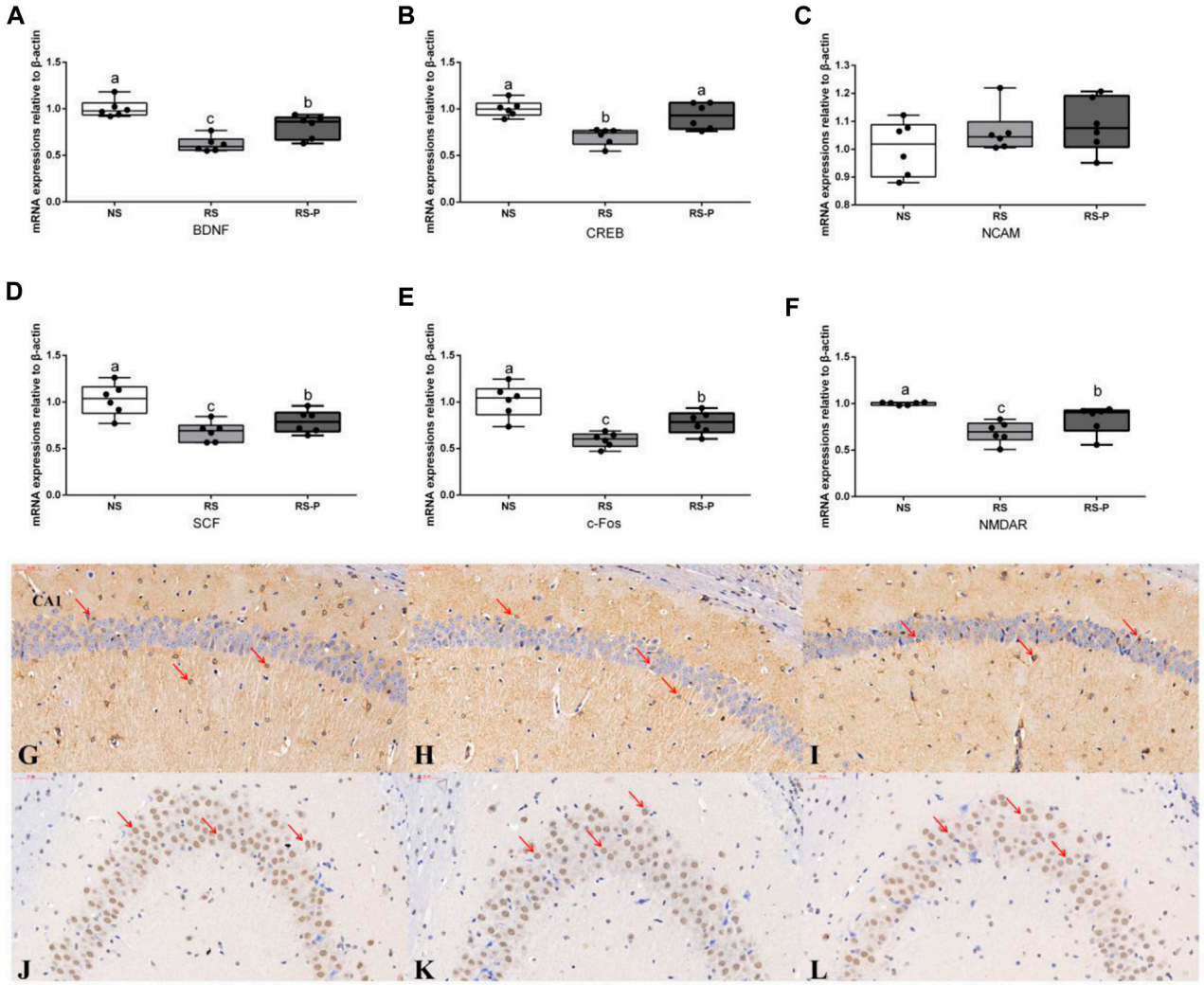

FIGURE 6 | Expression levels of memory-related functional proteins in the hippocampus. **(A)–(F)**: Relative expression of BDNF, CREB, NCAM, SCF, c-Fos, and NMDAR, respectively. Data are presented with the means ± standard deviation (n = 6). Bars with different letters are significantly different on the basis of Duncan's multiple range test ($P < 0.05$). **(G)–(I)** Immunohistochemistry of BDNF expressions in the hippocampus of NS **(G)**, RS **(H)** and RS-P **(I)** groups. **(J)–(L)** Immunohistochemistry of CREB expressions in the hippocampus of NS **(J)**, RS **(K)** and RS-P **(L)** groups. The BDNF- and CREB-positive cells are brown like the arrow indication, the magnification of all the figures are 40×. BDNF, brain-derived neurotrophic factor; CREB, cyclic ampresponse element binding protein; NCAM, neural cell adhesion molecule; SCF, stem cell factor; NMDAR, N -methyl-D-aspartate receptor.

Moreover, the protein content and mRNA expression level of IL-10 (**Figure 13F**) were lower ($P < 0.05$) in the RS group than those in the NS and RS-P groups without significance ($P > 0.05$).

Gut Microbiota

Microbial populations in the cecum were quantified via qPCR. The results are presented in **Figure 14**. The population of total bacteria (**Figure 14A**) was not significantly different ($P > 0.05$) among all experimental groups in the jejunum and ileum. However, both in the jejunum and ileum, the population of *Lactobacillus johnsonii* (**Figure 14B**) was significantly higher ($P < 0.05$) in RS-P group than those in NS and RS groups, while no significant difference was observed ($P > 0.05$) between NS group and RS group.

DISCUSSION

In modern society, psychological stress is common and negatively influences people's physiological system toward a low utility state (Guo et al., 2017). A quarter of the population in the United States is under high physiological stress (Oken et al., 2015). Stressful events can damage memory performances, such as memory consolidation and memory retrieval. (Mo et al., 2013) reported that increased stress susceptibility in animal models could cause a direct negative effect on memory function. Studies revealed negative influences on the majority of determined indexes, indicating that psychological stress could bring considerable harm to human health. Research achievements in different fields such as psychiatry, gastroenterology, and neuroscience

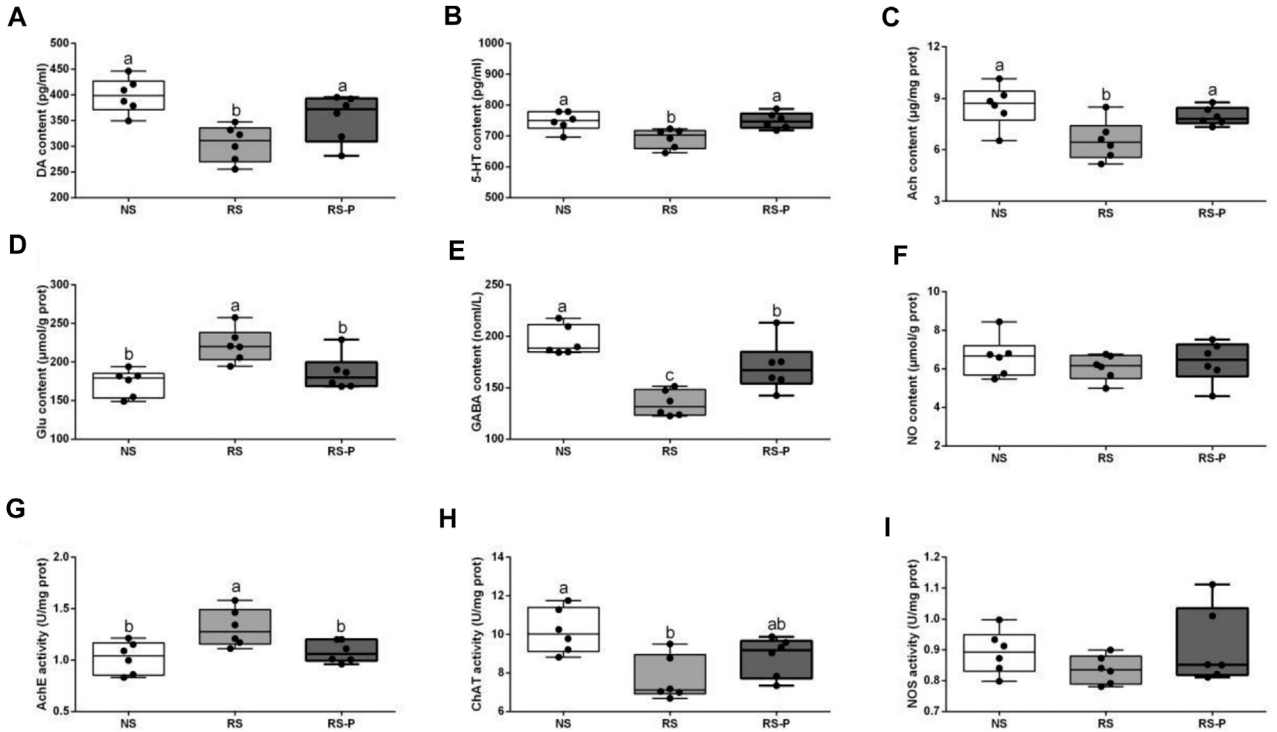

FIGURE 7 | Levels of neurotransmitters in the hippocampus. Data are presented with the means ± standard deviation (n = 6). Bars with different letters are significantly different on the basis of Duncan's multiple range test ($P < 0.05$). **(A)–(F)**: Levels of DA, 5-HT, Ach, Glu, GABA and NO, respectively; **(G)—(I)**: Activities of AchE, ChAT and NOS, respectively. DA, dopamine; 5-HT, 5-hydroxytryptamine; Ach, acetylcholine; Glu, glutamic acid; GABA, gamma-aminobutyric acid; NO, nitric oxide; AchE, acetyl-cholinesterase; ChAT, choline acetyltransferas; NOS, nitric oxide synthase.

are essential in advancing our understanding of GBA and exploring psychobiotics to regulate the brain by improving gut health, including intestinal inflammation and permeability. Many probiotics have psychobiotic potential of restoring or preventing hippocampal-dependent memory deficits in rodents induced by many different factors such as aging (Jeong et al., 2015) and diabetes (Davari et al., 2013). Although the antinociceptive (Iwakabe et al., 1998) and antidepressant (Ait-Belgnaoui et al., 2009) effects of probiotics on RS-induced mice have been reported, to our best knowledge, limited information is available regarding the preventive effects and underlying mechanism of potential psychobiotics on RS-induced memory dysfunction in rodents.

RS is one of the most widely utilized methods to mimic psychological stress. Rodents are isolated from their group with their movement confined to a restricted area (Bali and Jaggi, 2015; Miyamoto et al., 2017). Sheridan et al., 1991 reported that their immunity against virus is substantially depressed, and IL-2 secretion is reduced in the spleens and mediastinal lymph nodes in RS mice. Similar to many other stressors, RS damages the neuronal morphology and hippocampal function and induces dendritic remodeling in the prefrontal cortex, resulting in the increase in anxiety-like behaviors in humans and animals (Shansky et al., 2009). RS-induced anxiety could disrupt the working memory and therefore is one of the causes of memory dysfunction (Shackman et al., 2006). Stress could be controlled by the activation of the hypothalamic–pituitary–adrenal axis and the subsequent release of stress hormones such as corticosterone (in rodents) that are important for memory ability. The present results revealed increased corticosterone levels in the serum caused by RS and were in agreement with those by Guimaraes and colleagues (1993) and Gregus et al., 2005. In addition, the serum corticosterone level was attenuated by *L. johnsonii* BS15 pretreatment. This phenomenon was associated with the improved performances of the RS-P group during the novel object test, T-maze test, and passive avoidance test. Three different behavioral tests were utilized to evaluate the preventive effects of *L. johnsonii* BS15 on RS-induced hippocampus-related memory dysfunction. The mice in RS-P group were free from damaged memory abilities to some extent, thus suggesting the positive influences of *L. johnsonii* BS15 as a potential psychobiotic strain. The results of total bacteria and *L. johnsonii* population in this study indicated that *L. johnsonii* BS15 is possibly able to colonize in small intestines (jejunum and ileum) of mice, which makes it possible for this probiotic strain to alter the intestinal environment and thus exerts beneficial effects against psychological stress. Although detecting more copies of the genome does not directly demonstrate that the microbe is growing in the gut/metabolically active, as the qPCR approach will amplify genomic matrial from dormant cells, the observed differences

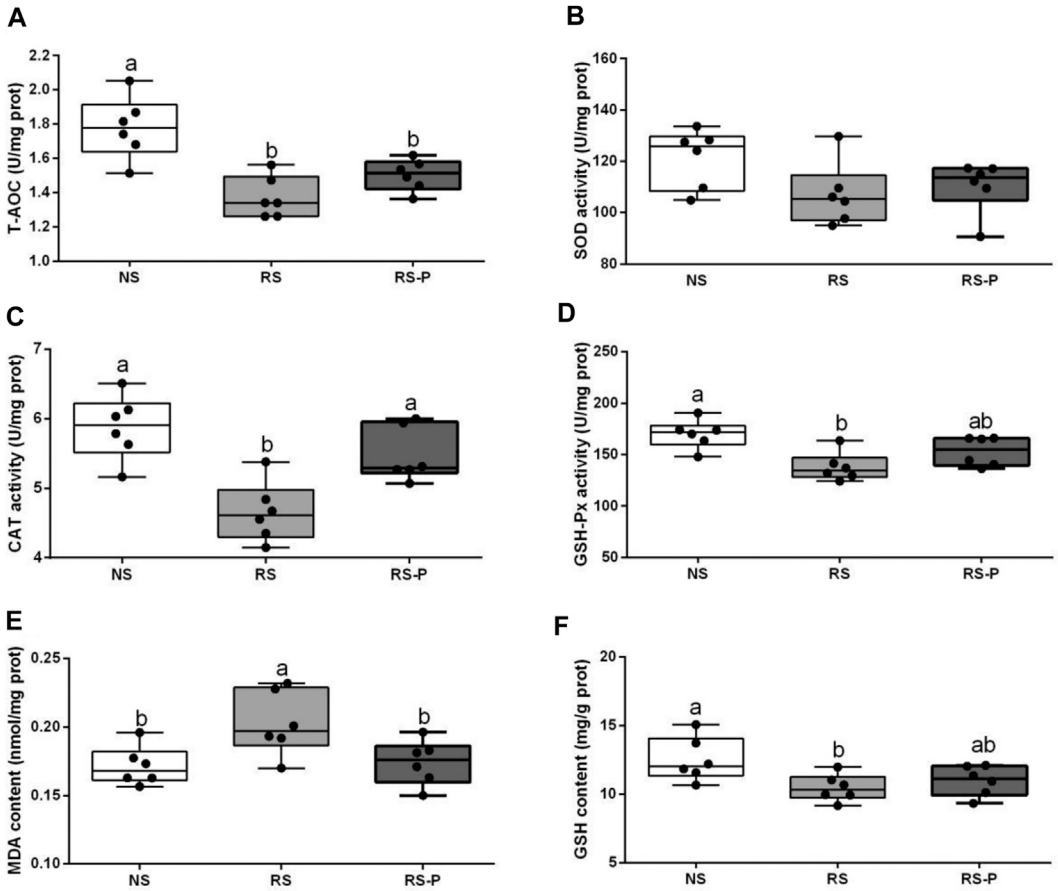

FIGURE 8 | Antioxidant indexes in the hippocampus. Data are presented with the means ± standard deviation (n = 6). Bars with different letters are significantly different on the basis of Duncan's multiple range test ($P < 0.05$). **(A)–(F)**: Activities or contents of T-AOC, SOD, CAT, GSH-Px, MDA and GSH, respectively. T-AOC, total antioxidation capacity; SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; GSH, glutathion.

between the groups still help to support our hypothesis to some extent.

The hippocampus is a crucial brain area for memory ability and is particularly susceptible to dietary (after less than 1 week) or psychological (30 min of psychological stresses) insult that could cause memory deficits (Guimaraes et al., 1993; Molteni et al., 2002). This work determined the changes of some important memory-related functional proteins, neurotransmitters, antioxidant capacity, and apoptosis-related functional proteins in the hippocampus to reveal the mechanism underlying the promising performances of *L. johnsonii* BS15 in behavioral tests for hippocampus-related memory abilities. Although the exact mechanism of stress-induced memory deficits remains unclear, the decreased neuroplasticity markers in the hippocampus are one of the most important proposed mechanisms (Reichelt et al., 2015).

Brain-derived neurotrophic factor (BDNF) plays an important role in the synaptic plasticity underlying the acquisition and/or consolidation of memory, and the hippocampus-specific deletion of BDNF could cause impaired spatial learning and novel object recognition (Heldt et al., 2007). A substantial decrease in the mRNA expression level of BDNF was found in the RS-induced mice, and this result agrees with the study by Xu et al., 2004. An enteric bacterial infection was reported by Gareau et al., 2011 to impair memory via reduced hippocampal BDNF; *Citrobacter rodentium*-infected mouse showed significant decreases in hippocampal BDNF levels, and reversal in BDNF expression was found in the probiotic-treated group. In addition, gut microbiota damaged by oral antimicrobials in mice reduces the hippocampal mRNA expression of BDNF, and this effect could be reversed by colonizing with normal microbiota (Bercik et al., 2011). A possible link between the significantly up-regulated mRNA levels of BDNF and the improved gut microbiota in RS-P group was suggested because a potentially harmful family of microorganisms, Enterobacteriaceae, was suppressed by *L. johnsonii* BS15. In addition, *Lactobacillus* spp. level also increased, indicating the probable suppression of other non-beneficial bacterial groups (Xin et al., 2014). The change of BDNF level may also be the major cause of the significant up-regulation of CREB. As one of the best-characterized transcription factors in the brain, CREB induced by BDNF is required for various memory forms and plays a role in neuronal resistance to insult in conjunction with BDNF (Gomez-Pinilla et al., 2002). SCF is reported to promote

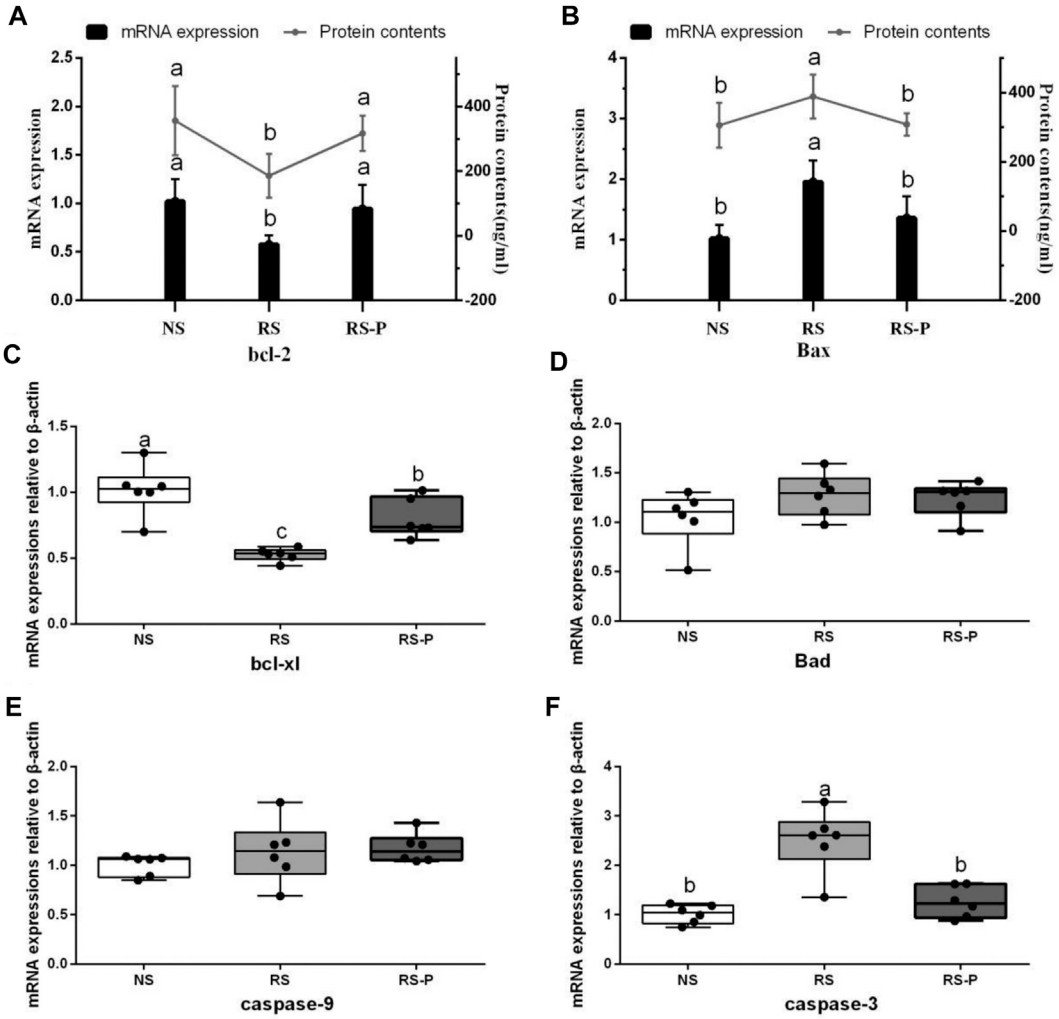

FIGURE 9 | Apoptosis-related functional protein contents and mRNA expression levels in the hippocampus. Data are presented with the means ± standard deviation ($n = 6$). Bars with different letters are significantly different on the basis of Duncan's multiple range test ($P < 0.05$). **(A)–(B)**: mRNA expression levels and protein contents of bcl-2 and Bax; **(C)—(F)**: mRNA expression levels of bcl-xl, Bad, caspase-9 and caspase-3, respectively.

neuronal plasticity (Hutchison et al., 2010). Given that the mRNA expression level of SCF was decreased by RS and effectively prevented by *L. johnsonii* BS15, the results of BDNF, CREB and SCF jointly revealed the close relationship between the preventive effects of *L. johnsonii* BS15 as a psychobiotic and the changes of neuronal plasticity. The results also showed that the RS-induced decreased mRNA expression of c-Fos and NMDAR, two functional proteins closely related to memory formation, were reversibly increased by *L. johnsonii* BS15, thereby suggesting its preventive effects against hippocampal-dependent memory dysfunction. The findings on c-Fos and NMDAR are consistent with the study by Wang et al., 2015 who applied *Lactobacillus fermentum* NS9 to protect the antibiotic-induced physiological and psychological abnormalities in rats.

Liang et al., 2015 pretreated rats with another potential psychobiotic strain, *Lactobacillus helveticus* NS8, and found that the DA and 5-HT contents in the hippocampus were substantially low in the chronic RS-induced group but were enhanced by NS8 pretreatment. Given that 5-HT and NE regulate mood and cognition, the results suggested the therapeutic potential of NS8 through the GBA. Similar results of DA and 5-HT contents in the hippocampus were found in the present study. Other crucial neurotransmitters were also determined. GLU and GABA are important for learning and memory in the hippocampus and serve as excitatory and inhibitory neurotransmitter (Tabassum et al., 2017). Increased Glu content and decreased GABA content commonly indicate damaged hippocampal functions and memory dysfunction. Therefore, *L. johnsonii* BS15 showed beneficial effects by protecting the memory abilities against RS by enhancing GABA content and decreasing Glu content in the RS-P group. Moreover, the preventive effects of *L. johnsonii* BS15 are revealed by the

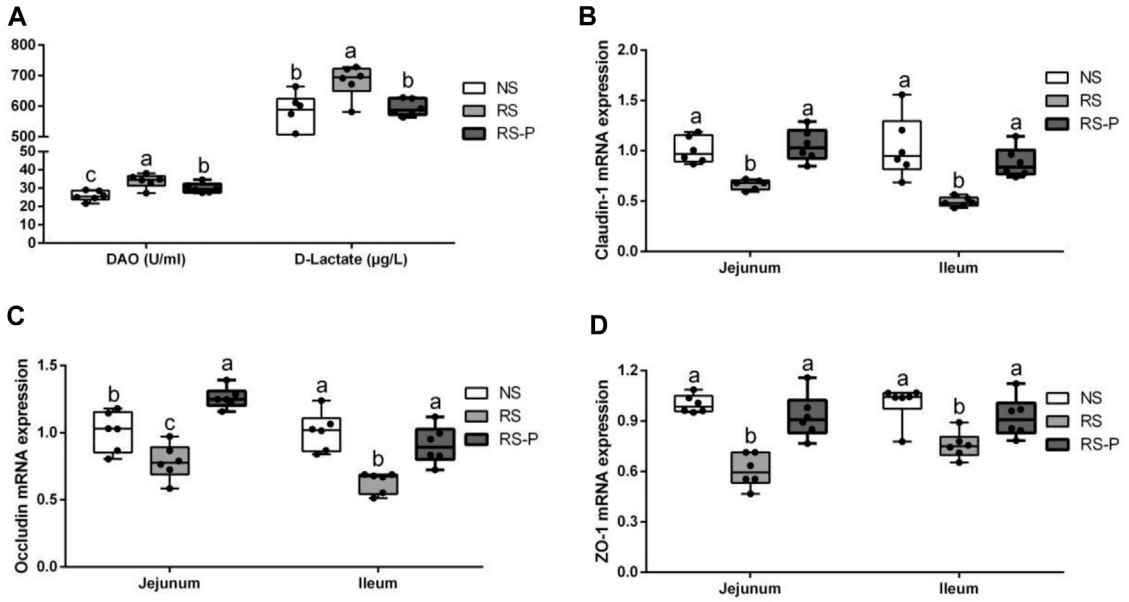

FIGURE 10 | Effect of L. johnsonii BS15 on gut integrity and permeability. Data are presented with the means ± standard deviation ($n = 6$). Bars with different letters are significantly different on the basis of Duncan's multiple range test ($P < 0.05$). **A**: Levels of DAO and D-Lactate in the serum. **(B)–(D)**: mRNA expression levels of tight junction protein (Claudin-1, Occludin and ZO-1, respectively) in the jejunum and ileum.

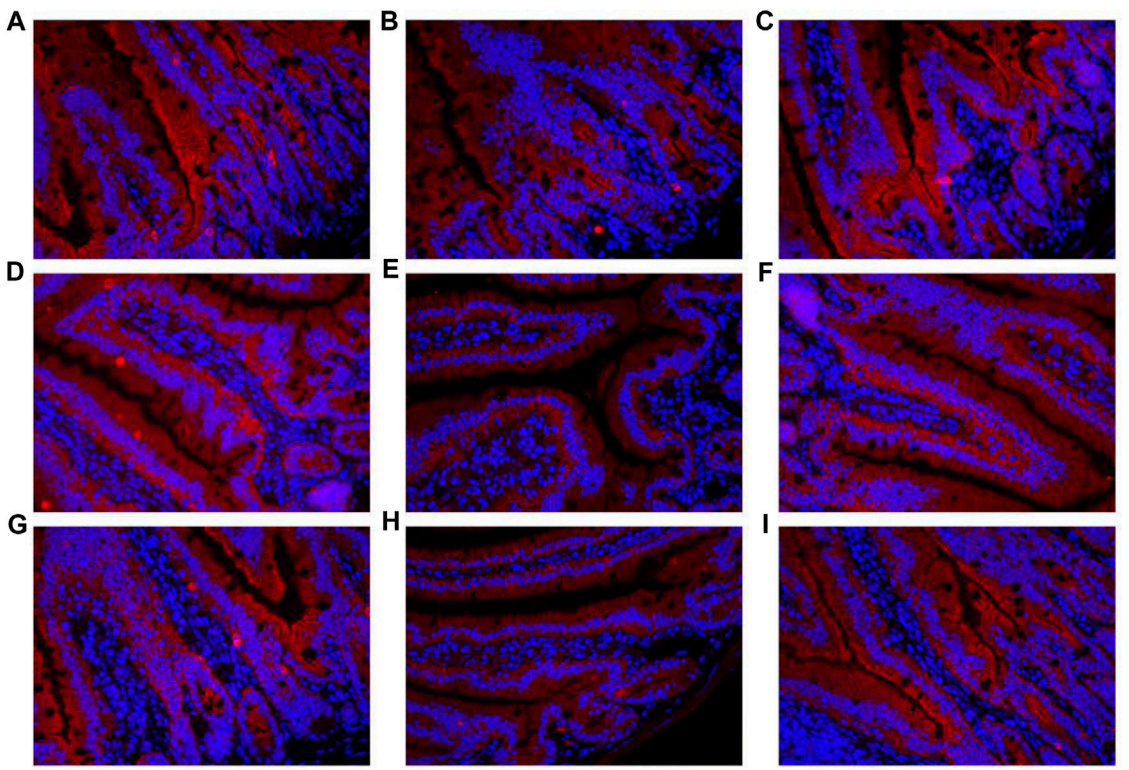

FIGURE 11 | Immunofluorescence of tight junction protein expressions in jejunum of mice. **(A)–(C)** Expressions of Claudin-1 in the jejunum of NS **(A)**, RS **(B)** and RS-P **(C)** groups. **(D)–(F)** Expressions of Occludin in the jejunum of NS **(D)**, RS **(E)** and RS-P **(F)** groups. **(G)–(I)** Expressions of ZO-1 in the jejunum of NS **(G)**, RS **(H)** and RS-P **(I)** groups. The tight junction proteins of. ZO-1-, claudin-1- and occludin are stained red, and the magnification of all the figures are 40×.

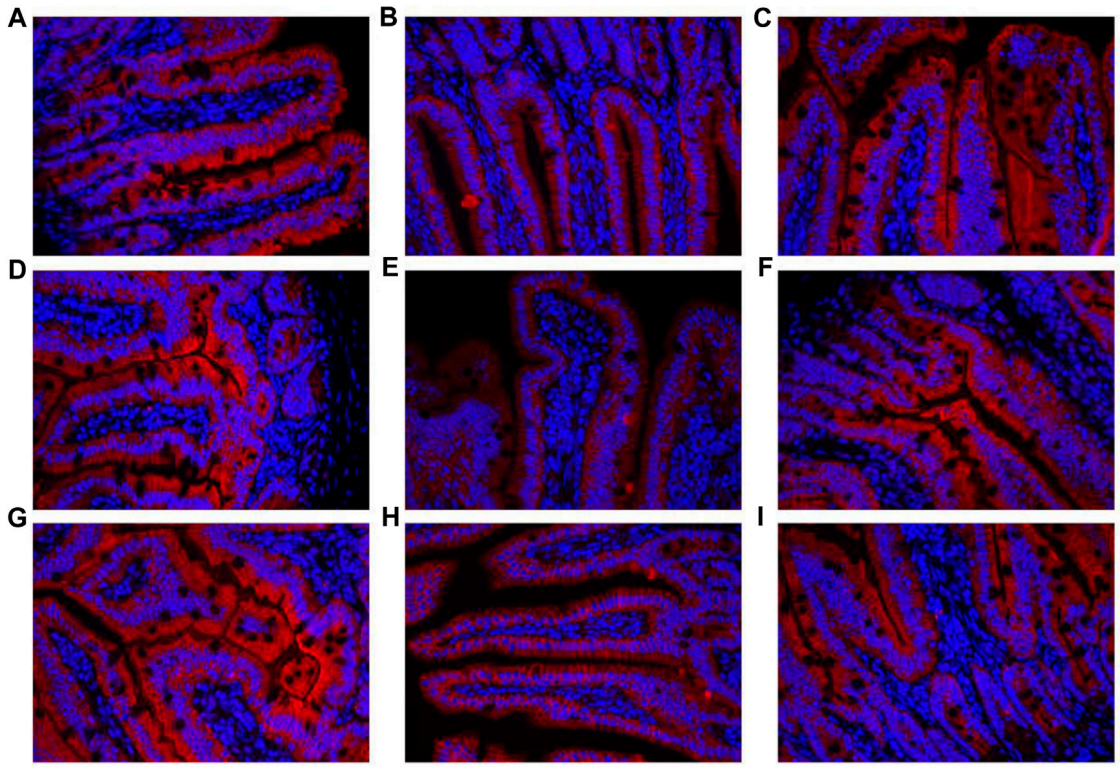

FIGURE 12 | Immunofluorescence of tight junction protein expressions in ileum of mice. **(A)–(C)** Expressions of Claudin-1 in the ileum of NS **(A)**, RS **(B)** and RS-P **(C)** groups. **(D)–(F)** Expressions of Occludin in the ileum of NS **(D)**, RS **(E)** and RS-P **(F)** groups. **(G)–(I)** Expressions of ZO-1 in the ileum of NS **(G)**, RS **(H)** and RS-P **(I)** groups. The tight junction proteins of. ZO-1-, claudin-1- and occludin are stained red, and the magnification of all the figures are 40×.

increase in Ach content, which also plays an important role in memory function, especially hippocampus-dependent learning (Haam and Yakel, 2017). Ach is catalyzed by ChAT and removed by the degradative function of AchE. The results indicated that RS could inhibit Ach accumulation by enhancing AchE activity and decreasing ChAT activity, thus damaging the memory function. Based on the results for the RS-P group, the changes of Ach could be prevented by *L. johnsonii* BS15.

Lipid peroxidation is an important process of molecular injury during various oxidative stresses causing hippocampal-dependent memory deficits. The production of reactive oxygen species generated by stress is responsible for lipid peroxidation indicated by increased MDA formation (Niki, 2012). CAT, SOD, and GSH-Px are antioxidant enzymes that protect against oxidative stress by degrading superoxide anions and hydrogen peroxide (Thakare et al., 2017). In this study, RS reduced the activities of T-AOC, GSH-Px, and CAT and increased MDA formation in the hippocampus, suggesting the enhancement of oxidative stress partly associated with the RS-induced memory dysfunction. This finding is consistent with the study by Freitas et al., 2014 and Thakare and colleagues (2017) who obtained similar results in the hippocampus of mice induced by RS for 7 h and 1 h, respectively. Molecular lesions could be induced by oxidative damage inducing and triggering apoptosis. Bcl-2 family proteins are located on the mitochondrial membrane, alter the permeability of mitochondrial membrane, and trigger apoptosis. High vulnerability to apoptotic activation could be indicated by increased Bax and low Bcl-2 (Kasprzak, 1995). The present study found the highest Bax and caspase-3 contents and lowest bcl-2 and Bcl-xL contents in the hippocampus of rodents in the RS group, suggesting that apoptosis mediated by mitochondria is remarkably activated by RS. *L. johnsonii* BS15 also effectively prevented the RS-induced side effects indicated by low Bax and caspase-3 and high Bcl-2 and Bcl-x in the RS-P group. Caspase-dependent apoptotic pathway can be activated by an imbalance between Bcl-2 and Bax, which results in high levels of caspase-3 and -9. Briefly, cytochrome c triggers the association of Apaf-1 to form an apoptosome by leaking out through the holes formed by Bax in the mitochondrial membrane; caspase-9 is activated through the apoptosome, which then triggers caspase-3 activation and consequently causes cell apoptosis (Jarskog et al., 2004). In the present study, the results of apoptosis-related proteins were related to the caspase-dependent apoptotic pathway and revealed that *L. johnsonii* BS15 pretreatment may inhibit oxidative damage in the hippocampus, modulate apoptosis level, and protect mice from RS-induced hippocampal-dependent memory deficits.

One of the most widely accepted mechanisms of how GBA influences cognitive functions is that bacteria in the gut initiate functional signals that are transmitted to the central nervous system through blood circulation. When the gut epithelium tight

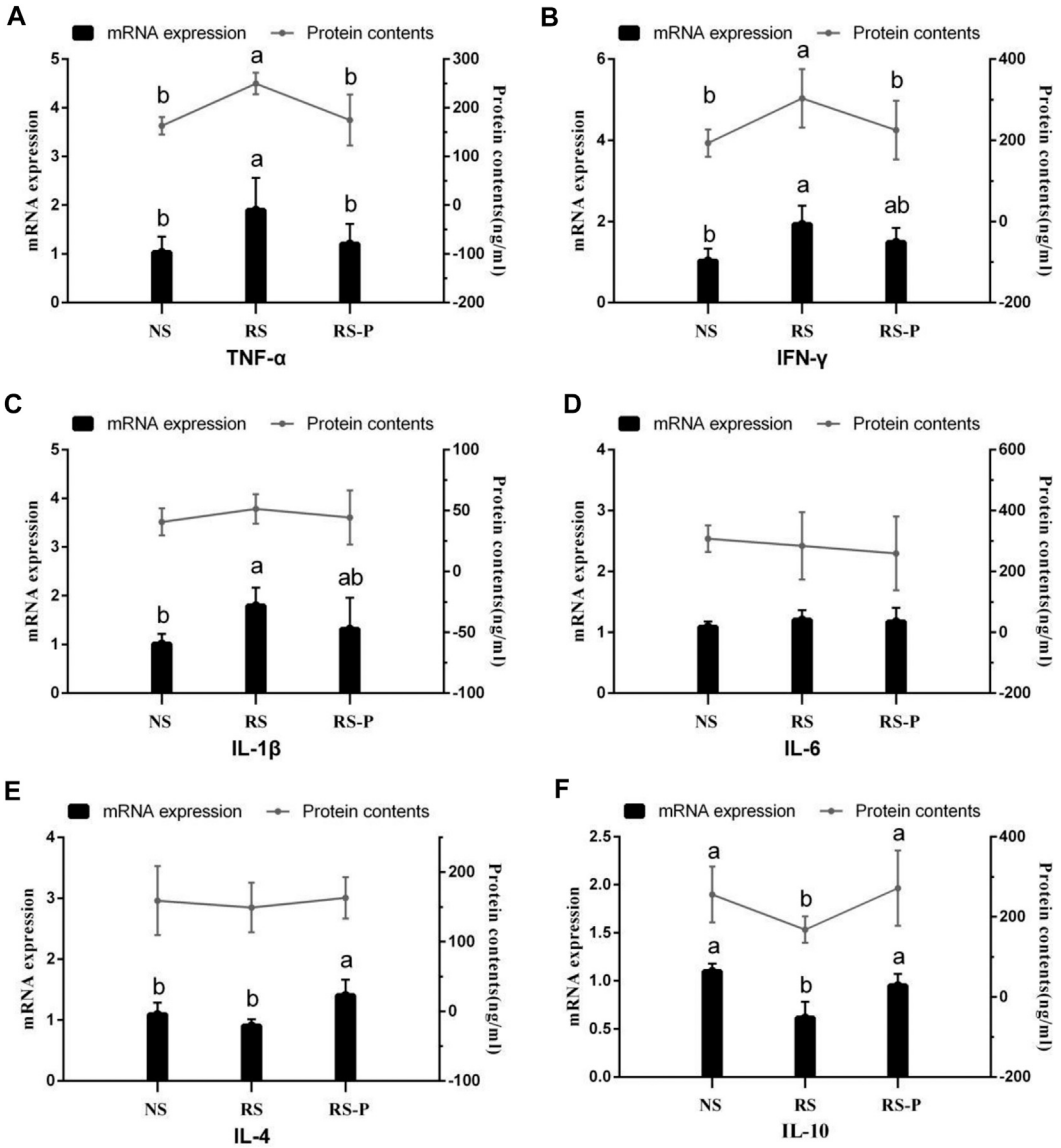

FIGURE 13 | Protein contents and mRNA expression levels of inflammatory factors in the ileum. Data are presented with the means ± standard deviation (n = 6). Bars with different letters are significantly different on the basis of Duncan's multiple range test ($P < 0.05$). **(A)–(F)**: mRNA expression levels and protein contents of TNF-α; IFN-γ; IL-1β; IL-6; IL-4; IL-10, respectively. TNF-α, tumor necrosis factor-alpha; INF-γ, interferon-gamma.

junctions are impaired, the damaged integrity of intestinal barrier becomes highly permeable, thus allowing the bacteria and/or their metabolites to easily enter the blood circulation (Sgritta et al., 2019). Therefore, the intestinal barrier protective effects of a potential psychobiotic may be the possible mechanisms to prevent mental diseases, including preserving the tight junction protein, inhibiting epithelial apoptosis, decreasing pathogenic bacterial adhesion, and reducing proinflammatory cytokines (Mennigen and Bruewer, 2009). Therefore, gut integrity and permeability were evaluated by determining DAO and D-lactate levels in the serum and the three key tight junction proteins in jejunum and ileum. The levels of DAO and D-lactate, circulating markers for the damage and repair of the intestinal mucosa, reflect the permeability and barrier function in the gut (Liu et al., 2017). The tight conjunction proteins (claudin-1, occluding, and ZO-1) created an intact layer of epithelial cells (Günzel and Yu, 2013). RS remarkably increased the release of DAO and D-lactate in the serum and decreased the mRNA expression levels of all three tight junction proteins, thus reflecting the RS-induced impairment of intestinal barrier. Therefore, *L. johnsonii* BS15 exerted beneficial effects on all determined indexes related to intestinal permeability except for the DAO level possibly through its protection for the intestinal epithelial cell membrane (Zeissig et al., 2007).

Proinflammatory cytokines, such as IFN-γ and TNF-α reduce the epithelial barrier function by influencing the epithelial tight

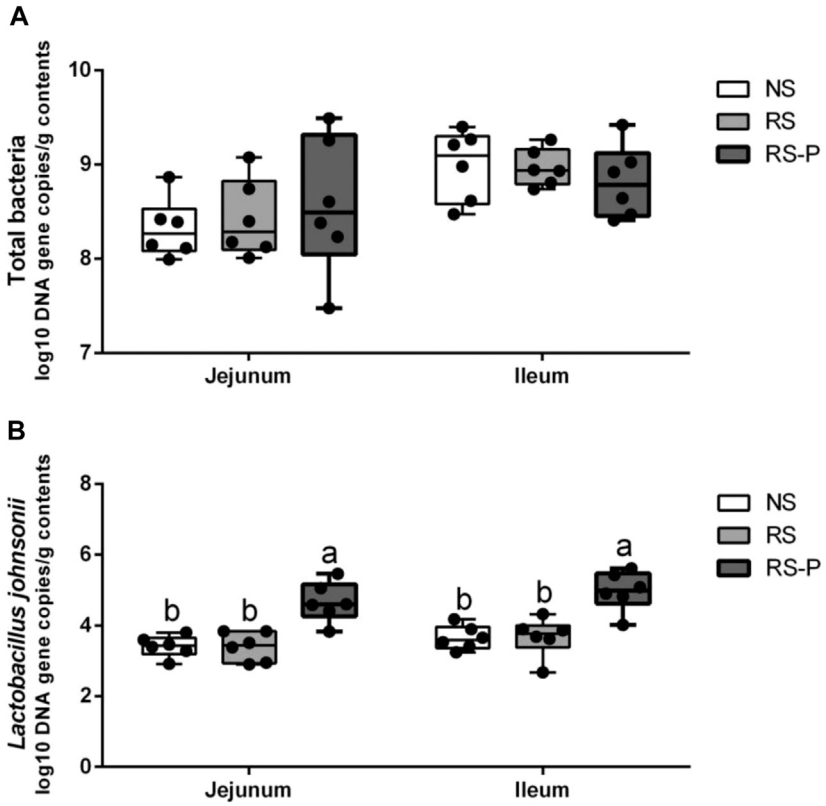

FIGURE 14 | Log10 DNA gene copies of total bacteria **(A)** and Lactobacillus johnsonii **(B)** as quantified by quantitative PCR in the jejunum and ileum. Data are presented with the means ± standard deviation (n = 6). Bars with different letters are significantly different on the basis of Duncan's multiple range test ($P < 0.05$).

junction and the induction of single cell apoptosis (Uwada et al., 2017). TNF-α and IFN-γ downregulate the mRNA expression of occludin and ZO-1, two tight junction proteins (Mankertz et al., 2000). Zeissig et al., 2004 also found that the damaged integrity of intestinal barrier could be restored to normal in humans by using TNF-α antibody therapy. According to our results, the impairment of intestinal barrier may be associated to the increased proinflammatory cytokines in the ileum. The highest mRNA expression levels of TNF-α, IFN-γ, and IL-1β, three important proinflammatory cytokines, were detected in the RS group, and the same trends of their protein contents (except IL-1β) were also observed. Moreover, IL-10, an anti-inflammatory cytokine, was inhibited by RS. The RS-induced changes were also observed by Gareau et al., 2008 in their study on the relationship between psychological stress and intestinal damage. Although *L. johnsonii* BS15 did not remarkably improve the proinflammatory cytokines, the determined anti-inflammatory cytokines (IL-4 and IL-10) were increased in the RS-P group compared with those in the RS group, indicating that *L. johnsonii* BS15 may induce preventive changes against RS by enhancing the intestinal anti-inflammatory effect and thus maintaining the intestinal integrity. These changes are in agreement with the results reported by Desbonnet et al., 2008 who administered another psychobiotic strain, *Bifidobacterium infantis,* to rats.

In conclusion, the pretreatment of *L. johnsonii* BS15 may prevent RS-induced hippocampus-related memory dysfunction by modulating intestinal inflammation and permeability, which indicated the psychoactive effects of *L. johnsonii* BS15 on positively influencing the GBA.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

All authors contributed to the design of the experiments. HW, JX, NS, LL, and XN performed the experiments. HW, SH, HM, and

YB analyzed and interpreted the results. SH, HW, TZ, DZ, and YB drafted and revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.662148/full#supplementary-material

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REVIEW
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Probiotic Supplementation for Rheumatoid Arthritis: A Promising Adjuvant Therapy in the Gut Microbiome Era

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Rheumatoid arthritis (RA) is a chronic immune-mediated inflammatory disease that ultimately leads to joint destruction and functional disability. Although the exact etiology of RA is not fully understood, it is well established that gut microbiota (GM) plays a vital role in the pathogenesis of RA, with accumulating evidence suggesting that gut dysbiosis induces a chronic inflammatory response that may be linked to disease development. Of interest, patients with RA have significant changes in the intestinal microbiota compared to healthy controls, and several studies have suggested the use of probiotics as a possible adjuvant therapy for RA. Benefits of probiotic supplementation were reported in animal models of arthritis and human studies, but the current evidence regarding the effect of probiotic supplementation in the management of RA remains insufficient to make definite recommendations. Several different strains of *Lactobacillus* and *Bifidobacteria*, as single species or in mixed culture, have been investigated, and some have demonstrated beneficial effects on disease activity in RA human subjects. As of now, *L.casei* probiotic bacteria seems to be the strongest candidate for application as adjuvant therapy for RA patients. In this review, we highlight the role of GM in the development and progression of RA and summarize the current knowledge on the use of probiotics as a potential adjuvant therapy for RA. We also review the proposed mechanisms whereby probiotics regulate inflammation. Finally, the role of fermented foods is discussed as a possible alternative to probiotic supplements since they have also been reported to have health benefits.

Keywords: dysbiosis, inflammation, autoimmunity, rheumatology, fermented foods

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INTRODUCTION

Rheumatoid Arthritis (RA) is a chronic immune-mediated inflammatory disorder that involves the synovial membranes of multiple joints (Sewell and Trentham, 1993; McInnes and Schett, 2017). The inflammatory process underlying this disease causes cartilage and bone destruction, damaging the joint structure and (Kang et al., 2017) leading to functional disability (Firestein, 2003; Smolen et al., 2016; Kalinkovich et al., 2018). In addition, systemic inflammation may impact other organs and

systems, such as the cardiovascular, pulmonary, skeletal bone, and brain (McInnes and Schett, 2011). RA is characterized by autoantibodies production in most patients, such as rheumatoid factor and anti-citrullinated protein antibodies (McInnes and Schett, 2011). Although the exact etiology of RA remains unknown, it has become evident that besides genetic factors, the environment (including the *internal* environment, the microbiome) plays a pivotal role in disease onset (Scherer et al., 2020).

RA patients have compositional and functional alterations in the gut microbiota (GM) (Zhang et al., 2015), and a significant decrease in microbial diversity compared with healthy controls has been reported (Chen et al., 2016). Moreover, the GM of RA patients exhibited decreased diversity with increased disease duration (Chen et al., 2016). *Faecalibacterium* is one of the most abundant *Firmicutes* in the human gut that produces butyrate, and a decreased abundance of *Faecalibacterium* and other butyrate producing taxa, such as *Flavobacterium*, have been reported in RA patients (Picchianti-Diamanti et al., 2018). On the other hand, the GM of RA patients has a significant increase in the order of *Lactobacillales* (Chen et al., 2016; Picchianti-Diamanti et al., 2018), and a higher variety of *lactobacilli* compared to healthy controls (Liu et al., 2013). Accordingly, an increase in the *Lactobacillaceae* family and the *Lactobacillus* genus in mice susceptible to collagen-induced arthritis (CIA) have been reported (Liu et al., 2016). Interestingly, the administration of some *Lactobacillus* species, seems to exert beneficial effects in RA clinical signs, which suggests that different *Lactobacilli* may have different roles in RA pathogenesis and disease activity modulation (Alipour et al., 2014; Vaghef-Mehrabany et al., 2014). In early RA patients, a significant increase of *Prevotella* genus has been frequently found in comparison to healthy controls, in particular *Prevotella copri* (*P. copri*) (Maeda et al., 2016; Paul et al., 2021; Reyes-Castillo et al., 2021). Given that the GM of RA patients differs from the general population and that anti-rheumatic drugs can exert positive effects on its regulation (Croia et al., 2019), microbiome research in the field of Rheumatology is expanding significantly (Manasson et al., 2020). Mounting evidence supports the existence of a reciprocal connection between drugs and GM, which can influence each other and have an impact on therapeutic outcomes (Bhat et al., 2017). Specifically, methotrexate (MTX) was shown to modify GM composition, partly restoring the microbial balance altered by the disease (Zhang et al., 2015; Picchianti-Diamanti et al., 2018). Moreover, the partial restoration of a beneficial microbiota induced mainly by anti tumor necrosis factor (TNF) drugs (as shown for etercept) can contribute to the clinical efficacy of these agents. A deeper understanding of the alterations occurring in the GM of patients on different therapeutic regimens could help set up individualized and supportive therapeutic strategies providing patients with more effective and safe care (Picchianti-Diamanti et al., 2018). In line with this, GM modulation and its interactions with the host have been reported as a strategy to prevent and control rheumatic diseases (Van De Wiele et al., 2016).

Probiotics are defined as *live microorganisms that, when administered in adequate amounts, confer a health benefit on the host* (Hill et al., 2014). Several studies have suggested the use of probiotics as a possible adjuvant therapy for RA patients (Ciccia et al., 2016; Wang et al., 2016; Reyes-Castillo et al., 2021). Various mechanisms whereby probiotics affect RA have been proposed, but are still poorly scientifically supported. Currently, most of the available research on this topic was conducted in animal models of arthritis. Exogenous bacteria can have a transient and subject-specific effect on the GM and, by its modification, can improve dysbiosis-related diseases (Zhang et al., 2016), such as RA. Since the probiotic effect is strain-dependent (Butel, 2014), the most appropriate strain must be chosen.

RA is a major global public health challenge with increasing age-standardized prevalence and incidence (Safiri et al., 2019). Despite the substantial advances with novel pharmacological therapies, the impact of RA on patient's functional capacity and quality of life remains a significant issue. Most patients have a chronic persistent form of the disease, as full remission is uncommon and sustained remission is even more unlikely to occur. Moreover, in order to control this disease, chronic treatment is needed, and multiple drug adverse effects often accumulate over the years. Indeed, there are still considerable unmet needs in RA management, and new safe treatment approaches that complement the existing ones are required (Smolen et al., 2016).

This paper aims to provide an up-to-date review of both animal and human studies investigating the effects of probiotics in RA and the proposed mechanisms whereby probiotics regulate inflammation. Since fermented foods can be used as a probiotic carrier and contain health-promoting components (Melini et al., 2019), we address their potential use in this context as a possible alternative to probiotic supplements.

GUT MICROBIOTA, IMMUNE SYSTEM AND RHEUMATOID ARTHRITIS

It is now well established that more than 100 trillion microorganisms, primarily bacteria, colonize the human oral-gastrointestinal tract, most residing in the distal intestine (Kamada et al., 2013). In recent years, there has been a dramatic increase in the interest regarding the composition and function of GM, resulting in a large body of evidence supporting GM as a crucial component in shaping host physiology and maintaining gut and immune homeostasis (Derrien and van Hylckama Vlieg, 2015).

The clinical picture of RA results from a complex interaction between various factors, including autoantibodies and signal transduction pathways of the innate and adaptive immune system (Croia et al., 2019). In RA patients, joint tissue is typically infiltrated by immune cells such as T cells, B cells, and macrophages, producing a variety of pro-inflammatory cytokines facilitating inflammation and eventually leading to tissue destruction (Volkov et al., 2020). Throughout life, GM plays a fundamental role in the induction, education, and function of the immune system, as well as the individuals' response to self-antigens (Belkaid and Hand, 2014; D'Amelio and Sassi, 2018; Wu and Wu, 2012). The modulation of GM may regulate the mechanism of gut immune tolerance, as it influences the number and function of colonic regulatory T cells (Tregs) (Tanoue et al., 2016). Tregs suppress inappropriate activation of effector T cells by secreting anti-inflammatory cytokines (Kalinkovich and Livshits, 2019; Kayama et al., 2020). On the other hand, the mucosal immune

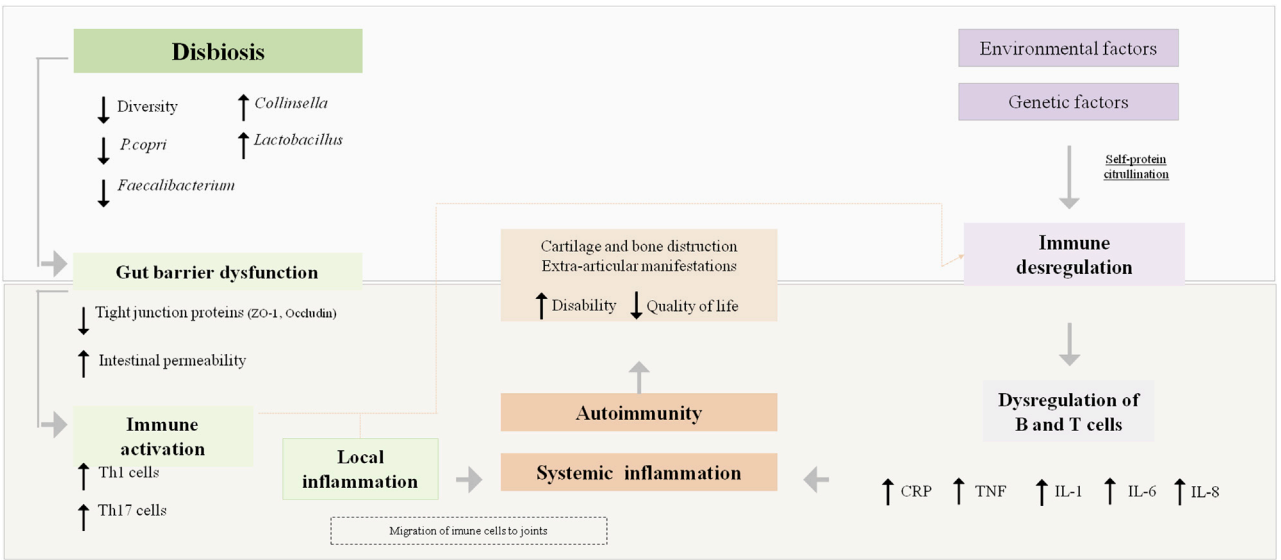

FIGURE 1 | Rheumatoid arthritis pathophysiology and the proposed mechanisms by which gut microbiota could influence its pathogenesis. CRP, C-reactive; P. Copri, Prevotella copri; ZO-1, Zonula occluclens-1; IL, Interleukin; TNF, tumour necrosis factor.

system has a crucial role in developing and maintaining a healthy GM (X. Wu et al., 2016). Due to this interdependent relationship, gut dysbiosis, a compositional and functional alteration of GM (Levy et al., 2017), may influence host susceptibility to many immune-mediated diseases such as RA, type 1 diabetes, multiple sclerosis, and systemic lupus erythematosus (de Oliveira et al., 2017). Additionally, there is emerging literature reporting on the role of changes in GM in the pathogenesis of chronic immune-mediated inflammatory disorders, including RA (Ciccia et al., 2016; Kalinkovich et al., 2018; Zhong et al., 2018). Deregulation of host responses as a consequence of gut dysbiosis could affect distant anatomical sites through the activation of host immune responses, and this could be the mechanism contributing to the onset of an idiopathic inflammatory condition like RA (Cho and Blaser, 2012).

To support the hypothesis that changes in GM composition play a significant role in the onset and progression of RA (Horta-Baas et al., 2017; Kang et al., 2017), several mechanisms by which GM is associated with arthritis have been proposed. These include regulating the host's immune system (triggering T cell differentiation), activating antigen-presenting cells (APCs) through an effect on Toll-like receptors (TLRs) or NOD-like receptors (NLRs), promoting the citrullination of peptides by enzymatic action, antigenic mimicry, and increasing the intestinal mucosal permeability (Horta-Baas et al., 2017; Guerreiro et al., 2018). Regarding the effect on the expression of TLRs of APCs, this may contribute to an imbalance in the Th17/Treg cell ratio and this local immune response could lead to systemic autoimmunity (Horta-Baas et al., 2017). Thus, the existing literature suggests that GM could contribute to or prevent the expansion of autoimmunity and inflammation during the preclinical and clinical phases of RA, and GM could influence transitions between these phases (Wilson et al., 2020). **Figure 1** summarizes the pathophysiology of RA and the proposed mechanisms whereby GM could participate in triggering autoimmunity and systemic inflammation in susceptible individuals.

Since the diet is an essential environmental factor impacting intestinal microbiota composition, increasing attention has been given to its role in the pathogenesis, progression, and activity of rheumatic diseases (Gioia et al., 2020). In this regard, the use of probiotic bacteria has been suggested as a possible strategy to correct gut dysbiosis and promote the homeostasis of a healthy microbiota, having an impact on systemic immune responses and thus could be used as adjuvant therapy to treat immune-mediated diseases (Gareau et al., 2010; de Oliveira et al., 2017).

MECHANISMS UNDERLYING PROBIOTICS EFFECTS ON GUT MICROBIOTA, IMMUNE SYSTEM AND RHEUMATOID ARTHRITIS

A link between the composition and activity of GM and human health and disease has been previously described (Azad, Sarker, Li, et al., 2018). Although the local effects of probiotic bacteria on gut health are well reported, the mechanisms behind their systemic anti-inflammatory and immunomodulating potential have not been wholly explored (Vieira et al., 2016; Plaza-Diaz et al., 2019; Oliviero and Spinella, 2020). A set of mechanisms whereby probiotics regulate inflammation have been postulated, which can be exerted not only via direct immune system modulation, but also through indirect mechanisms (La Fata et al., 2018; Kalinkovich and Livshits, 2019).

Probiotic's Direct Mechanisms of Immune System Modulation

Specific probiotic bacteria modulate the immune response by affecting different cells involved in innate and acquired immunity, such as epithelial cells and dendritic cells (DCs), natural killer cells (NK), macrophages, and lymphocytes (Bermudez-Brito et al., 2012; La Fata et al., 2018; Cristofori et al., 2021).

The innate immune system develops the primary response to pathogens after activation of the pattern recognition receptors (PRRs), which are expressed on immune and non-immune cells, such as NK cells, DCs, macrophages, fibroblasts, and epithelial cells (Bermudez-Brito et al., 2012; Plaza-Diaz et al., 2019; Cristofori et al., 2021). Toll-like receptors (TLRs) are the most widely studied PRRs, which can activate signaling pathways that affect cell proliferation and cytokine production to modulate the immune system (Ferlazzo et al., 2011; Cristofori et al., 2021). It is well established that probiotics can downregulate TLR expression, reducing inflammation (Gómez-Llorente et al., 2010).

Concerning the adaptive immune response, T cells are central to immune balance (Plaza-Diaz et al., 2019). Inflammatory responses driven by T helper (Th)1 and Th17 cells protect the host from pathogens, but their overactivation is linked to the pathogenesis of detrimental inflammation. The adaptive immune cells Foxp3+ Tregs suppress inappropriate activation of effector Th cells by secreting anti-inflammatory cytokines, such as IL-10, modulating the immune response (Kalinkovich and Livshits, 2019; Peters et al., 2019; Kayama et al., 2020). Probiotics have also been reported to influence cytokine production by APCs, which initiates adaptive responses (Azad, Sarker, and Wan, 2018). Beyond the described immunomodulatory properties involving DCs and T cells, some probiotic strains also have a role in increasing the production of secretory IgA once they promote the differentiation of B cells into plasma cells (Liu, Tran, et al., 2018). Secretory IgA provides a defense against pathogens by limiting bacterial adhesion to the epithelium and preventing the penetration of host tissue (Azad, Sarker, and Wan, 2018; Liu, Tran, et al., 2018).

Probiotic's Indirect Mechanisms of Immune System Modulation

Probiotics can also interact with the host immune system through indirect mechanisms, which involve the modulation of GM. The mechanisms by which probiotic strains have been proposed to modulate GM include regulating the gut epithelial barrier and the mucus layer characteristics, secretion of antimicrobial compounds and competition with pathogenic bacteria (Vieira et al., 2016; Jethwa and Abraham, 2017; Cristofori et al., 2021).

The gut epithelium, which separates the luminal environment from the intestinal milieu, has a key role in assuring the permeability to nutrients and other molecules, as well as blocking the entry of toxins and other microorganisms (Deane et al., 2017; Van Spaendonk et al., 2017). Tight Junction (TJ) proteins, located in the apical part of the intestinal epithelial cells, are crucial elements to ensure the functionality and integrity of the mucosal barrier (Ulluwishewa et al., 2011; Lee, 2015). When an alteration in the expression or localization of TJ proteins occurs, the epithelial barrier function is compromised due to increased permeability (Ulluwishewa et al., 2011). The use of probiotics and the consequent increase in the short-chain fatty acids (SCFA) release, particularly butyrate, has been reported to enforce the gut barrier function as butyrate strengthens the barrier through increased expression of TJ components zonula occludens (ZO)-1, ZO-2, and cingulin (Bordin et al., 2004; Deane et al., 2017; Liu, Tran, et al., 2018).

The intestinal epithelium is covered by a viscoelastic mucus layer, mainly composed of mucins, high-molecular-weight glycoproteins produced by goblet cells (La Fata et al., 2018). Mucins are responsible for building a protective barrier containing digestive enzymes, promoting food passage, and at the same time prevent the entry of bacteria into the lamina propria by blocking their adhesion to the epithelial cells (Corfield et al., 2000; Derrien et al., 2010; De Santis et al., 2015). The intestinal mucus layer has a primary role in protecting against mechanical, chemical, and biological attacks to the gut and contributes to the maintenance of intestinal homeostasis (Paone and Cani, 2020). Some probiotic strains have been reported to regulate mucin expression, altering the properties of the mucus layer and indirectly regulating the gut immune system (La Fata et al., 2018). Examples include the adherent *Lactobacillus spp*, which can stimulate MUC3 expression in human intestinal epithelial cells and MUC2 production and secretion (Sicard et al., 2017; Bron et al., 2017), and *Lactobacillus reuteri* (*L. reuteri*), which has a protective effect against dextran sulfate sodium-induced colitis in mice, increasing the mucus layer thickness (Ahl et al., 2016).

Specific probiotic-modulated local and systemic metabolites have been reported to have anti-inflammatory and antimicrobial functions, such as SCFA, dietary tryptophan, adenosine, and histamine (Liu, Alookaran, et al., 2018). One of the primary mechanisms by which probiotics compete in this environment is through competitive exclusion, by which they adhere to the intestinal mucosa and prevent the subsequent entry of pathogens into the lamina propria (Liu, Tran, et al., 2018; Santis et al., 2015). Moreover, the adhesion of probiotic microorganisms to epithelial cells may trigger a signaling cascade, leading to immunological modulation (Markowiak and Slĩ, 2017). As previously described, SCFAs exert an indirect anti-inflammatory effect through improving intestinal barrier function (Bodkhe et al., 2019; Kolodziejczyk et al., 2019). Butyrate is particularly relevant in modulating inflammation, as it inhibits histone deacetylase and regulates the expression of numerous pro-inflammatory genes, inducing the differentiation and expansion of Tregs and regulating cytokine production (Koh et al., 2016; Liu et al., 2021; Liu, Tran, et al., 2018; Peters et al., 2019).

Probiotic's Mechanisms and the Pathophysiology of Rheumatoid Arthritis

Crosstalk between gut epithelium, immune system, and commensal bacteria is key to starting the systemic inflammatory response (Y. Liu, Tran, et al., 2018). An imbalance between anti-inflammatory and pro-inflammatory cytokines, including interleukin (IL)-1β, TNF, interferon (IFN)-γ, IL-6, IL-12, and IL-17, plays a central role in the inflammatory processes involved in the pathogenesis of RA (Smolen and Steiner, 2003; So et al., 2008; Amdekar et al., 2011).

The proposed mechanism for the gut-joint axis in inflammatory arthritis is related to the hyperpermeability of the gut wall, which leads to the exposure of immune system to microorganisms, leading to a systemic immune response that triggers a local inflammatory process within the joints (Jethwa and Abraham, 2017; Liu et al., 2021).

Considering the perturbed GM as a pivotal trigger in the pathogenesis of RA, interest has emerged regarding the clinical interest of probiotics to correct gut dysbiosis and downregulate the pro-inflammatory cytokine cascade implicated in inflammatory arthritis (Wang et al., 2016; Mohammed et al.,

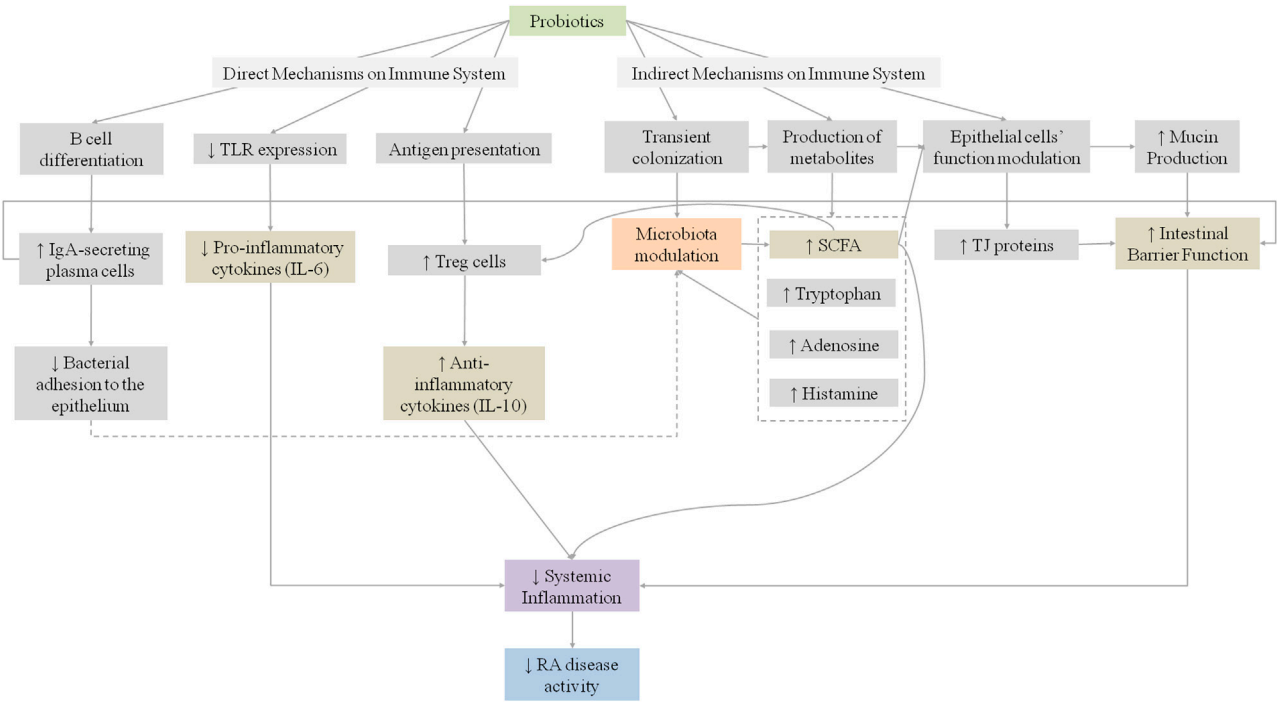

FIGURE 2 | Proposed mechanisms for the influence of probiotics on systemic inflammation in rheumatoid arthritis patients. IgA, Immunoglobulin A; IL, Interleukin; RA, Rheumatoid; SCFA, short Chain Fatty Acids; TJ, Tight Junctions; TLR, Toll-like receptors.

2017; Lowe et al., 2020). Probiotics upregulate regulatory cytokines produced by Tregs or tolerogenic DCs in the gut. Cytokines travel to target organs and expand Tregs that traffic to inflammation sites (Marietta et al., 2016). Probiotic-driven metabolic products, such as SCFA, also impact immune response and systemic inflammation by regulating immune cell function (Oliviero and Spinella, 2020). SCFA as regulators of several leukocyte functions including production of eicosanoids and chemokines and cytokines, such as TNF, IL-2, IL-6 and IL-10, exhibit anti-inflammatory properties (Vinolo et al., 2011; Kalinkovich and Livshits, 2019). Moreover, probiotic bacteria and its metabolic products can keep a balance between tolerance to the intestinal microflora and resistance against harmful bacterial colonization, adherence, and translocation (Mohammed et al., 2017). These properties may be useful to correct the hyperpermeability of gut wall proposed for the gut-joint axis in inflammatory arthritis.

A meta-analysis of randomized trials investigating the effect of *Lactobacillus* as single species or in mixed cultures with *Bifidobacterium species* concluded that probiotic supplementation reduced serum levels of IL-6 (Mohammed et al., 2017). Another systematic review and meta-analysis that investigated the effectiveness of *Lactobacillus casei (L. casei)* supplementation in RA reported that a significant reduction of C-reactive protein (CRP) was achieved with this specific strain (Rudbane et al., 2018). Studies have also reported that *L. casei* might help alleviate RA symptoms and suppress pro-inflammatory cytokines in individuals undergoing treatment with disease-modifying anti-rheumatic drugs (DMARDs), which suggests a positive synergistic effect between DMARDs and probiotics on arthritis (Alipour et al., 2014; Pan et al., 2017, 2019).

These findings suggest that the administration of probiotic bacteria may have a beneficial effect on the inflammatory activity of RA, through the regulation of cytokine production, improvement of the intestinal barrier function, and its positive synergistic effect with DMARDs. **Figure 2** summarizes the proposed mechanisms for the influence of probiotics on RA.

EXPERIMENTAL EVIDENCE OF PROBIOTIC EFFECTS ON RHEUMATOID ARTHRITIS PREVENTION AND TREATMENT

Experimental Evidence From Animal Models

This section will review the current evidence for microbiome manipulation by using probiotic bacteria in animal models of arthritis for both disease prevention and treatment.

Studies With the Collagen-Induced Arthritis Animal Model

Several studies have proposed a link between GM and CIA development with oral administration of several bacterial strains in mice.

A study conducted by Fan et al. compared the effects of a preventive and therapeutic treatment with *Bifidobacterium adolescentis* (*B. adolescentis*) in CIA rats (Fan et al., 2020). Preventive *B. adolescentis* administration had better results in

reducing the clinical symptoms, rebalancing the pro- and anti-inflammatory responses, and reversing the gut dysbiosis than late *B. adolescentis* treatment. Early probiotic administration performed better in promoting SCFAs production, had significant higher Tregs cells frequency and lower levels of TNF compared to the late *B. adolescentis* treated group. Moreover, SCFA positively correlated with Tregs and negatively correlated with pro-inflammatory cytokines in the early treated group (Fan et al., 2020). These findings suggest that the introduction of *B. adolescentis* before arthritis can impact the onset of arthritic inflammation, and support that GM manipulating therapies should be provided at an early stage of the disease or even before disease occurrence.

Another study investigated the effects of the oral administration of *L. casei Shirota* (LcS) in a mouse model of CIA (Kato et al., 1998). LcS administration during induction of CIA suppressed the abnormal anti-type II collagen antibody production and delayed onset and reduced severity of CIA. It was concluded that oral administration of LcS reduced the humoral and cellular immune responses to CIA, which could result in reduced rates of CIA development in mice (Kato et al., 1998).

Yamashita et al. evaluated the effect of oral administration of *L. helveticus* SBT2171 in reducing the incidence and progression of CIA (Yamashita et al., 2017). Oral administration of *L. helveticus* SBT2171 significantly relieved joint swelling and suppressed weight loss. These findings suggested that *L. helveticus* SBT2171 can downregulate the abundance of immune cells and the production of anti-type II collagen antibodies and IL-6, suppressing CIA symptoms indicating its potential for use in the prevention of RA (Yamashita et al., 2017).

Amdekar et al. assessed the therapeutic efficacy of *L. casei* in a CIA model of arthritis and reported a therapeutic effect of this probiotic when administered after the onset of arthritis (Amdekar et al., 2011). There was a significant reduction in the arthritis score with a significantly decreased secretion of pro-inflammatory cytokines (TNF and IL-6) and an increased concentration of IL-10, an anti-inflammatory cytokine (Amdekar et al., 2011). The authors suggested that the exerted anti-inflammatory effect of *L. casei* was a result of Cyclooxygenase (COX)-2 and NF-κB inactivation (Amdekar et al., 2011). Previous research on the COX-2 have already sugested that it has a key role in inflammation in RA (Kang et al., 1996), as COX-2 has been pointed out as the responsible for the overproduction of prostaglandins. Prostaglandins are implicated in different phases of inflammatory reactions, and its synthesis is down regulated by anti-inflammatory cytokines, such as IL-10. In this regard, the proposed mechanism provided by Amdekar et al. for the obtained effect with L. casei in a CIA model, involves prostaglandins inhibition due to an increased secretion of IL-10 promoted by the probiotic treatment.

Another study from Amdekar et al. evaluated the anti-inflammatory and antioxidant properties of *L. casei* and *Lactobacillus acidophilus* (*L. acidophilus*) as a therapeutic protocol in an experimental model of CIA. The results suggested that *L. casei* and *L. acidophilus* exhibit antiarthritic and anti-inflammatory properties by suppressing IL-6, TNF, IL-17, and IL-1β production and upregulating IL-10 and IL-4 (Amdekar et al., 2013). Along with the reported imbalances between pro-inflammatory and anti-inflammatory cytokines, which have been reported to play an important role in initiation and pathogenesis of arthritis, prostaglandins, nitric oxide, and reactive oxygen species (ROS) are also released at the site of inflammation in many rheumatic diseases, damaging the cartilage and the components of extracellular matrix. In this study, *L. casei* and *L. acidophilus* significantly decreased lipid peroxidation and catalase (CAT) levels, and increased the concentration of glutathione peroxidase (GPx), glutathione (GSH) and superoxide dismutase (SOD) (Amdekar et al., 2013). These findings are particularly relevant as they suggest that the beneficial effects of *L. casei* and *L. acidophilus* are due, not only to their anti-inflammatory effect, but also to their antioxidant properties.

In a study conducted by Marietta et al., an isolated human gut commensal *Prevotella histicola* (*P. histicola*) was tested for treating CIA in HLA-DQ8 transgenic mice in prophylactic and therapeutic protocols (Marietta et al., 2016). Mice were monitored for the onset and progression of CIA. Treating mice with *P. histicola* significantly decreased the incidence and severity of arthritis compared to controls (Marietta et al., 2016). The microbial modulation of arthritis was dependent on the generation of Tregs in the gut, resulting in suppression of Th17 response and increased release of IL-10. Moreover, treatment with *P. histicola* improved intestinal barrier function by increasing the expression of TJ proteins, ZO-1 and occludin (Marietta et al., 2016).

So et al. investigated the effect of *L. casei* in suppressing the inflammatory immune responses of RA by testing its impact on the effector functions of $CD4^+$ T cells (So et al., 2008). This study demonstrated that *L. casei* could effectively suppress RA-related pathways by simultaneously down-regulating Th1 effector functions and upregulating anti-inflammatory IL-10 (So et al., 2008). Additionally, oral administration of *L. casei* suppressed arthritis, reduced hind paw swelling, lymphocyte infiltration and the destruction of cartilage tissue. Several reports have demonstrated the beneficial effects of *Lactobacillus* species in mouse models of arthritis however further research is needed to describe the mechanisms underlying its efficacy.

Animal studies support the thesis that *L. casei* strains down-regulate immune-system function (Vaghef-Mehrabany et al., 2018), which is beneficial in the case of RA and other inflammatory diseases. Results from a number of preclinical studies have demonstrated that various strains of *L. casei* can be effective in reducing arthritis score and decreasing serum inflammatory cytokines in RA (Kato et al., 1998; So et al., 2008; Amdekar et al., 2011, 2013; Pan et al., 2019). Although there are numerous studies reporting the efficacy of Lactic Acid Bacteria (LAB), their underlying mechanisms of action are still to be fully elucidated. Proposed mechanisms for *L. casei* strains include the improved proportion of Th2/Th1 cytokines, including the induction of Treg cells and down-regulation of Th1 cells. Once bound to TLR 2/6, *L. casei* bacteria, trigger various intracellular mechanisms, which will ultimately contribute to the maturation of FoxP3-$CD4^+$ towards FoxP3 Treg cells (Vaghef-Mehrabany et al., 2018). Tregs cells are the main producers of IL-10, providing inhibitory effects on Th1 cells

(Issazadeh-Navikas et al., 2012). Amdekar *et al.*, have also proposed a similar mechanism for *Lactobacillus* species (Amdekar et al., 2013). Therefore, the altered cytokine balance in favour of anti-inflammatory cytokines seems to be the main mechanism of action of probiotics in RA and should be the basis of future research. The role of antioxidant effects should be further elucidated.

Studies With the Adjuvant-Induced Arthritis Animal Model

Concerning the role of probiotic bacteria on disease progression, Pan et al. investigated the potential of administrating *L. casei* in the treatment of adjuvant-induced arthritis (AIA) and reported profound changes of microbial species in the gut as well as alterations in clinical signs during arthritis induction and progression phases, such as inhibition of joint swelling, lower arthritis scores, and prevention of bone destruction (Pan et al., 2019). Thirty days after prophylactic treatment with *L. casei*, a significant reduction in pro-inflammatory cytokines levels was observed. Moreover, an increased relative abundance of several *Lactobacillus* strains was also detected, such as *L. acidophilus, Lactobacillus hominis, L. reuteri, and Lactobacillus vaginalis*, suggesting that *L. casei* improves arthritis mainly through establishing the rebalance of the *Lactobacillus* strains (Pan et al., 2019). Some *Lactobacillus* strains have been reported to drive T cell differentiation from intraepithelial $CD4^+$ T cells into immunoregulatory Treg. Also, their metabolic products, such as SCFAs, influence colonic Treg cell homeostasis (Smith et al., 2013; Cervantes-Barragan et al., 2017).

Rovensky et al. investigated the efficacy of *Escherichia coli O83* (Colinfant®) in the treatment of AIA (Rovenský et al., 2009). They studied the effect of Colinfant® alone, of Colinfant® in combination with MTX and MTX alone. They found a significant reduction in both inflammation and arthritis-associated alterations (reduction of hind paw swelling and arthrogram score) with MTX and with the combination of MTX and Colinfant® (Rovenský et al., 2009). They also reported a more significant improvement of the arthritis score with combination treatment than with MTX alone. However, the use of Colifant® alone had no impact on inflammatory markers (Rovenský et al., 2009).

Another study using an AIA model investigated whether *B. coagulans* and inulin, administered either isolated or in combination, influenced arthritis severity in rats (Abhari et al., 2016). A significant clinical improvement was observed in *B. coagulans* and/or inulin treated rats. This improvement included suppression of paw swelling and a decrease in pro-inflammatory parameters, such as fibrinogen and TNF-α (Abhari et al., 2016).

A study conducted by Achi et al. evaluated three strains of *Bifidobacteria*, namely *Bifidobacterium breve* NCIM 5671 (*B. breve* NCIM 5671), *Bifidobacterium longum* NCIM 5672, and *Bifidobacterium bifidum* NCIM 5697, to investigate their prophylactic effect in an AIA model (Achi et al., 2019). The results have demonstrated that *Bifidobacteria* can reduce the severity and progression of arthritis. However, *B. breve* NCIM 5671 had better antiarthritic effects in the rat model than the other bifidobacterial species studied, suggesting that the effect is strain-dependent, and these strains should be further explored for their putative positive impact on RA treatment (Achi et al., 2019).

Table 1 summarizes the characteristics and main findings regarding probiotic effects on RA in preclinical studies.

Summary of Evidence From Animal Models

Even after several decades of research, RA remains a complex disease of unknown etiology and without a cure (Choudhary et al., 2018). Animal models are widely used for testing potential new therapies for RA, and despite their recognized limitations, it is evident that these have provided valuable information regarding RA pathogenesis and the underlying mechanisms of disease. When considering all existing animal models of arthritis, the most commonly found question is which model is most predictive of therapeutic efficacy in human subjects with RA, as each model features a different mechanism driving disease expression (Hegen et al., 2008). Considering the problem to be investigated, the benefits of each model should be closely evaluated in order to make the most appropriate choice. Accordingly, for the identification and validation of drug targets, AIA and CIA models have great reproducibility and are the most commonly used models (Choudhary et al., 2018). The AIA model is characterized by acute inflammation and severe destruction, useful for the evaluation of the early structural consequences of arthritis and also for studies of pain pathways (Boissier and Bessis, 2017; Vidal et al., 2018). On the other hand, CIA has been an extremely popular model since its conception, once its underlying mechanisms involve numerous elements of the innate and adaptive immune systems, making it a useful model both for developing concepts to be extended to human subjects and for validating new treatment targets (Boissier and Bessis, 2017). The breach of tolerance and generation of auto antibodies towards self, are recognized as the most important characteristics of the CIA model, which makes it a very good *in vivo* model for RA studies (Asquith et al., 2009).

We have considered both prophylactic (when probiotic administration started before immunization or before arthritis onset) and therapeutic (when dosing with study probiotic started after clinical signs of disease) treatment regimens with probiotic bacteria in CIA and AIA models. A number of preclinical studies reported the beneficial effects of probiotics via multiple pathways, including restoring the dysbiosis of GM in a prophylactic way (Achi et al., 2019; Pan et al., 2019; Fan et al., 2020). A study conducted by Liu et al., has reported significant differences in the microbiome composition of CIA-susceptible and CIA-resistant mice (Liu et al., 2016). When transplanted to germ-free mice, the microbiome of the CIA-susceptible mice aggravated CIA disease severity, suggesting a relationship between GM composition and CIA susceptibility (Liu et al., 2016). These results showed that intestinal microbiota strongly affects the balance between pro- and anti-inflammatory immune responses in CIA. Although several studies reported differences in the microbiome composition of RA when compared to controls, little is known about the highly personalized microbiome dynamics during the induction, progression, and treatment of arthritis. The genus *Lactobacillus* is significantly more abundant prior to arthritis onset in CIA-susceptible mice than in CIA-resistant mice (Liu et al., 2016), However, results obtained by Pan et al., indicated that *L. casei* could influence the disordered microbiome and ameliorate arthritis via modulation of *Lactobacillus* strains

TABLE 1 | Probiotic effects on animal models of arthritis.

Author, year	Study objective	Animal model/ administration timing	Probiotic strain	Administration dose	Evaluated parameters	Main findings
Animal models of CIA						
Kato et al. (1998)	To investigate the effects of *L. casei Shirota* on the development of CIA and immune responses	Male DBA 1 mice/after arthritis modeling	*L. casei* Shirota	PG1: 0.25 × 10^9 CFU/day PG2: 0.5 × 10^9 CFU/day PG3: 1 × 10^9 CFU/day PG4:2 × 10^9 CFU/day	Arthritis score; incidence of CIA; serum anti-CII antibodies; IFN-γ; IL-4	*L. casei Shirota* ↓ arthritis incidence in all groups; ↓ arthritis severity; ↓ CII-specific antibodies IgG2a and IgG2b; ↓ IFN-γ
So et al. (2008)	To investigate how *L. casei* suppresses the progression of CIA	Female lewis rats/before and after arthritis induction	*L. casei*	5 × 10^9 CFU/dose	Paw swelling; arthritis score; CII-specific antibodies; CII-reactive pro-inflammatory molecules	*L.casei* ↓ hind paw swelling; ↓ lymphocyte infiltration; ↓ destruction of cartilage tissue; ↓ IL-1β; ↓ IL-2; ↓ IL-6; ↓ IL-12; ↓ IL-17; ↓ IFN-γ; ↓ TNF; ↓ COX-2; ↑ IL-10; ↓ serum CII-specific IgG2a and IgG2b; ↓ T cell proliferation (in both the pretreatment and acute phase treatment)
Amdekar et al. (2011)	To investigate the therapeutic efficacy of *L. casei* in a CIA model	Female wistar rats/after arthritis induction	*L.casei* ATCC 334	2 × 10^8 CFU/ml	Arthritis score; serum cytokines; hind knee joint morphology	*L. casei* ↓ arthritis score; ↓ IL-6; ↓ TNF-α; ↓ infiltration of neutrophils in joint; ↓ bone erosion; ↓ pannus formation
Amdekar et al. (2013).	To evaluate antioxidant and anti-inflammatory potential of *L. casei* and *L. acidophilus* in a CIA model	Male wistar rats/after arthritis induction	*L.casei* ATCC 334 *L.acidophilus* ATCC314	PG1: 2 × 10^8 CFU/ml (*L. casei*) PG2: 2 × 10^8 CFU/ml (*L. acidophilus*)	Arthritis score; serum cytokines; oxidative stress markers (GSH, CAT, SOD, lipid peroxidation, GPx)	*L. casei* and L. acidophilus ↓ arthritis score; ↑ IL-4; ↑ IL-10; ↓ IL-6; ↓ TNF; ↓ IL-1β; ↓ IL-17; ↓ CAT; ↓ lipid peroxidation; ↑ GSH; ↑ GPx; ↑ SOD
Marietta et al. (2016)	To evaluate the effects of *P. histicola* for treating CIA	DQ8 mice/before and after arthritis induction	*P. histicola*	1 × 10^9 live bacteria	Arthritis incidence; arthritis onset; arthritis severity; expression of TJ proteins; serum cytokines	*P. histicola* ↓ incidence of arthritis; ↓ severity of arthritis; ↓ IL-2; ↓ IL-17; ↓ TNF; ↑ IL-4; ↑ IL-10; ↓ anti-CII antibodies; ↓ gut permeability; ↑ ZO-1
Yamashita et al. (2017)	To evaluate the effect of *L. helveticus* on the development of CIA, antibody production and immune cells	Male DBA 1J mice/after arthritis induction	*L.helveticus* SBT2171	PG1:1.2 × 10^{10} CFU/g (oral administration) PG2: (Intraperitoneal inoculation)	Hind limb joint tissues; serum CII-specific antibodies; serum cytokines; total immune cells	*L. helveticus* oral administration ↓ joint swelling; ↓ body weight loss; ↓ serum CII-specific IgG and IgG1; *L. helveticus* intraperitoneal inoculation ↓ arthritis incidence; ↓ joint damage; ↓ serum IL-6; ↓ total B-cells; ↓ $CD4^+$ T cells in the inguinal LNs
Fan et al. (2020)	To investigate the effects of *B. adolescentis* before and after arthritis induction on GM composition and immune responses	Female wistar rats/before and after arthritis induction	*B. adolescentis* cocktail including 5 strains HuNan2016-7-2 AHWH4-M1 FSDJN3Q1 M1DZ09M1 FSDJN12W5	5 × 10^9 CFU/ml/day (per strain)	Ankle thickness; arthritis score; serum cytokines; serum anti-CII antibodies; tregs in MLNs; level of TJ proteins; GM composition; faecal SCFA	Preventive *B. adolescentis* performed better in ↓ ankle thickness; ↓ arthritis score; ↓TNF; ↑ Tregs in MLNs; ↑ SCFAs; ↑ mRNA level of ZO-1 and occludin; maintaining the gut microbial communities similar to the CG
Animal models of AIA						
Rovenský et al. (2009)	To evaluate the effect of *E. coli* O83 on AIA during basal treatment with MTX	Male lewis rats/after arthritis induction	*E. coli* O83	8 × 10^8 bacteria/ml (1 ml/kg body mass)	Body mass; hind paw swelling; arthrogram score; serum albumin	E.*coli* O83 + MTX ↓ hind paw swelling; ↓ arthrogram score
Abhari et al. (2016)	To investigate the possible influence of *Bacillus coagulans* on immune responses and disease progression	Male wistar rats/before and after induction	*Bacillus coagulans*	10^9 spores	Paw thickness; Fn; SAA; TNF-a; a1AGp	Pretreatment with *Bacillus coagulans* ↓ Fn; ↓ SAA; ↓ TNF

(Continued on following page)

TABLE 1 | (*Continued*) Probiotic effects on animal models of arthritis.

Animal models of CIA						
Author, year	**Study objective**	**Animal model/ administration timing**	**Probiotic strain**	**Administration dose**	**Evaluated parameters**	**Main findings**
Achi et al. (2019)	To evaluate the prophylactic effect of B. breve NCIM 5671, B. longum NCIM 5672 and B. bifidum NCIM 5697 in a rat model of arthritis	Male wistar rats/before and after induction	*B. breve* NCIM 5671 *B. longum* NCIM 5672 *B. bifidum* NCIM 5697	10^8–10^9 cells/0.5 ml	Paw volume; bone mineral content; oxidative stress markers; antioxidant enzyme activity; serum cytokines; eicosanoids; expression of COX2	*Bifidobacterium* strains ↓ paw volume; ↓ PGE2; ↓ LTB4; ↓ LTC4; ↓ IL-1β; ↓ TNF; ↓ IL-6; ↓ MCP1; ↑ IL-4; ↑ IL-10; ↓ COX2 expression
Pan et al. (2019)	To evaluate the effect of *L. casei* for the treatment of arthritis	SD rats/after arthritis induction	*L. casei* ATCC334	2×10^8 CFU/day	Hind paw volume; arthritis score; serum cytokines; GM composition	*L. casei* ↓ hind paw volume; ↓ arthritis score; ↓ IFN-γ; ↓ TNF; ↓ IL-1β; ↓ IL-17; ↓ IL-6; rebalance of the *lactobacillus* species; maintains the redox balance of oxidative stress

a1AGp, alpha-1-acid glycoprotein; AIA, adjuvant-induced arthritis; B, bifidobacterium*; CAT, catalase; CFU, colony-forming units; CG, control group; CIA, collagen-induced arthritis; CII, type II collagen; COX-2, Cyclooxygenase-2; E,* escherichia*;; Fn, fibrinogen; GM, gut microbiota; GPx, glutathione peroxidase; GSH, glutathione; IFN, interferon; IL,* interleukin*; L,* lactobacillus*; LNs, lymph nodes; Tregs, regulatory T cells; MLNs, mesenteric lymph nodes; MTX, methotrexate; PG, probiotic group; SAA, serum amyloid A; SCFA, short chain fatty acids; SOD, superoxide dismutase; TJ, tight junction; TNF, tumour necrosis factor and ZO-1, zonula occludens-1.*

(Pan et al., 2019). These findings highlighted the importance of monitoring changes in microbial communities during disease progression and provided powerful evidence to explain the evolution of the GM in RA. Of interest, Fan et al. reported the beneficial effects of early treatment in maintaining gut microbial communities (Fan et al., 2020).

There is no model fully reproducing a human rheumatic disease, which means that therapeutic interventions in animal models only provide partial information (Bessis et al., 2017). However, the development of novel treatment interventions for RA still relies on the careful analysis of studies in animal models combined with clinical observations.

Experimental Evidence From Human Studies (Randomized Controlled Trials)

Randomized Controlled Trials With *Lactobacillus rhamnosus*

The effect of probiotic supplementation was studied in stable RA in a randomized controlled trial (RCT) evaluating the treatment with *Lactobacillus rhamnosus (L. rhamnosus)* GG versus placebo (Hatakka et al., 2003). Patients were not under treatment with DMARDs, but most of them were on stable medication with glucocorticoids (GC, 75% in the probiotic group and 62% in the placebo group) and non-steroidal anti-inflammatory drugs (NSAIDs, 75% in the probiotic group and 77% in the placebo group). In this study, the intervention group was given two capsules of *L. rhamnosus* (ATCC 53103) GG (Gefilus®, Valio Ltd.; $\geq 5 \times 10^9$ colony-forming units (CFU) per capsule), twice a day, for 12 months, and the placebo group received identical capsules without the bacteria. There were no statistical differences in the clinical parameters, biochemical variables, and Health Assessment Questionnaire (HAQ) between groups. Inflammatory parameters were not significantly reduced. Interestingly, although there were no statistical differences in disease activity, more subjects in the intervention group reported subjective well-being when compared to the placebo group (Hatakka et al., 2003).

Supplementation with *L. rhamnosus* combined with *L. reuteri* was also studied as adjunctive therapy for patients with active RA (Pineda et al., 2011). In this study, patients on stable medication (for at least one month) with DMARDs, steroids and/or NSAIDs were randomized to receive one capsule taken twice daily, for 3 months or placebo. The probiotic group received a supplement containing *L. rhamnosus* GR-1 and *L. reuteri* RC-14 (containing 2×10^9 CFU/capsule). The placebo group received a capsule containing the same ingredients without the bacteria. A decrease in serum levels of IL-1a, IL-6, IL-10, IL-12p70, and TNF was reported, but placebo caused a significantly greater decline in the production of IL-6, IL-12p70, and TNF, as well as IL-15, IL-17. Finally, although there was a significant improvement in the HAQ score in the probiotic group, no between-group differences were found (Pineda et al., 2011).

Randomized Controlled Trials With *Lactobacillus casei*

A different strain of *Lactobacillus, L. casei,* was also studied for its potential benefits in RA. In the study conducted by Vaghef-

Mehrabany et al., patients with inactive to moderate RA (i.e., a disease activity score (DAS28) of <5.1) who were following a stable medication regimen for at least three months were included; current medication for most patients included DMARDs and GCs, but not NSAIDs or biologics. The probiotic group received a daily capsule of *L. casei* 01 (>10^8 CFU/capsule) for eight weeks (Vaghef-Mehrabany et al., 2014). The placebo group received capsules containing only maltodextrin (the excipient used in the probiotic capsules). The probiotic supplementation significantly decreased three of the assessed serum pro-inflammatory cytokines (TNF, IL-6, and IL-12). Moreover, a significant increase in serum levels of IL-10 was observed in the probiotic group. In this study, the pain visual analogue scale (VAS) score decreased, compared with baseline, by 43.96% in the probiotic group and by 5.99% in the placebo group at the end of the study (Vaghef-Mehrabany et al., 2014). Regarding the effects of probiotic supplementation with *L. casei* on oxidative stress, Vaghef-Mehrabany et al. conducted a secondary analysis and concluded that this intervention had no significant effect on the oxidative status of patients with RA compared to placebo (Vaghef-Mehrabany et al., 2016). Additionally, the same authors found that this intervention with *L. casei* 01 also significantly decreased serum high sensitivity C-reactive protein (hs-CRP), global health score (assessed by VAS), DAS-28, as well as tender and swollen joint counts (Alipour et al., 2014). Regarding disease activity, DAS-28 (mean ± standard deviation) decreased from 2.56 ± 1.01 at baseline to 2.07 ± 0.82 at the end of the study in the probiotic group, while a much smaller improvement in DAS-28 was observed in the placebo group (2.31 ± 0.90 at baseline to 2.23 ± 0.86 at the end of the study) (Alipour et al., 2014).

Zamani et al. tested *L. casei* combined with other strains (Zamani et al., 2016). In this RCT, the intervention group received, in addition to their conventional medications (DMARDs and GCs), a daily capsule containing *L. casei* (2 × 10^9 CFU/g), *L. acidophilus* (2 × 10^9 CFU/g) and *B. bifidum* (2 × 10^9 CFU/g) for eight weeks, and the placebo group took capsules filled with cellulose for the same amount of time. Although this trial was conducted in patients with moderate to severe disease activity (DAS-28 > 3.2), contrarily to the previous studies, probiotic supplementation also resulted in improved DAS-28. In the probiotic group, DAS-28 (mean ± standard deviation) decreased from 4.0 ± 0.7 at baseline to 3.7 ± 0.7 at the end of the trial, while the decrease in the placebo group was only from 4.1 ± 0.7 at baseline to 4.0 ± 0.7 at the end of the trial. The authors also found a significant decrease in serum hs-CRP concentrations and other parameters not previously studied, such as serum insulin levels (Zamani et al., 2016). In line with Vaghef-Mehrabany et al., this intervention did not influence biomarkers of oxidative stress compared with the placebo among patients with RA (Zamani et al., 2016).

Lastly, *L. casei* was also tested combined with other strains in the study conducted by Cannarella et al. (Cannarella et al., 2021). In this trial, RA patients in the probiotic group took a daily sachet for 60 days containing 10^9 CFU/g of each of the following strains: *L. casei* LC-11, *L. acidophilus* LA-14, *Lactococcus lactis* LL-23, *B. lactis* BL-04 and *B. bifidum* BB-06, and the placebo group took maltodextrin daily for the same amount of time. Similarly to the previous studies, the usual medication was maintained in both groups during the experiment. The probiotic group showed a significant reduction in white blood cell counts, TNF and IL-6 plasma levels, but this intervention did not alter DAS-28 (median of 3.83 at baseline vs a median of 3.88 at the end of the study in the placebo group; median of 3.20 at baseline vs a median of 3.18 at the end of the study in the probiotic group). Moreover, no differences were found in the IL-10, adiponectin, CRP and erythrocyte sedimentation rate (ESR) between groups. The authors also assessed parameters that were not reported in the previous studies, such as oxidative/nitrosative profile and antioxidant defences. They found that probiotic supplementation improved the oxidative/nitrosative profile and increased the antioxidant defences in patients with RA. In this regard, the probiotic group showed lower nitric oxide metabolites, and higher sulfhydryl group and a total radical-trapping antioxidant parameter compared to the placebo group (Cannarella et al., 2021).

Randomized Controlled Trials With *Bacillus coagulans*

An RCT with *B. coagulans* GBI-30, 6,086® (GanedenBC30®), also used in combination with DMARDs, was conducted by Mandel et al. in RA patients. In this trial, besides the probiotic itself, the preparation included green tea extract, methylsulfonylmethane, vitamins and minerals, and the placebo contained microcrystalline cellulose. There was a statistically significant improvement in the pain scale compared to placebo (Mandel et al., 2010). Besides, the probiotic supplementation resulted in a reduction of CRP and greater improvement in patient-global assessment, self-assessed disability, the ability to walk two miles and participation in daily activities compared to placebo (Mandel et al., 2010). The authors did not report DAS-28 but suggested that *B. coagulans* GBI-30, 6,086® supplementation may be an effective adjunct therapy for the relief of RA symptoms. Trials assessing disease activity are warranted.

Summary of Evidence From Randomized Controlled Trials

Overall, there is some evidence from human RCTs that probiotic supplementation can improve disease activity and the inflammatory status of RA patients when used in addition to the patient's conventional medications. Additionally, the recently published RCT by Cannarella et al. reported that probiotic supplementation could also increase the antioxidant defences and improve oxidative/nitrosative profile in RA patients (Cannarella et al., 2021). **Figure 3** summarizes the experimental evidence for the use of probiotic bacteria in RA according to taxonomic distribution.

Several different strains have been tested for their benefit in RA patients, as single species or in mixed culture, in different dosages, which is a limitation that could explain conflicting results. Selecting the most appropriate strain for administration to RA patients is of major importance, and this should be sought in future research projects. As of now, probiotic supplementation with *L. casei* seems to be the strongest candidate to be used as adjuvant therapy for RA patients, and current evidence suggests a minimum of 10^8 CFU/capsule per day to obtain significant results. Supporting this, a systematic review and meta-analysis investigating the effectiveness of probiotic

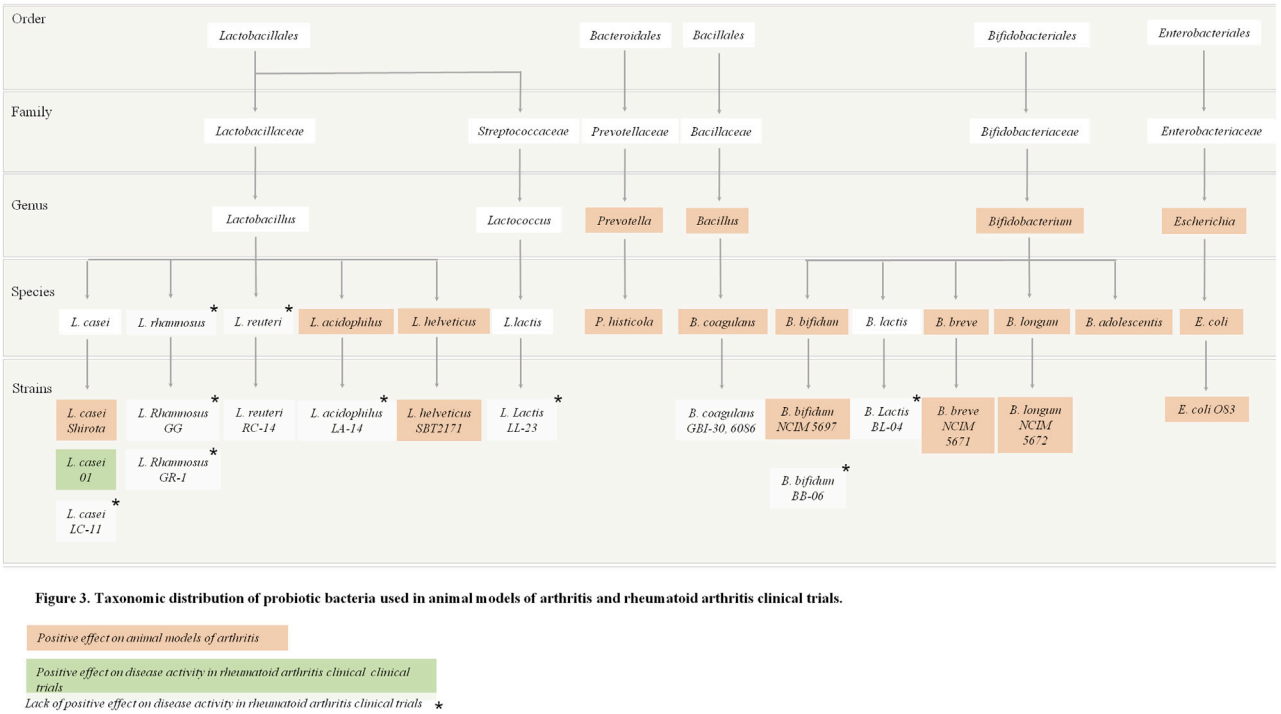

FIGURE 3 | Taxonomic distribution of probiotic bacteria used in animal models of arthritis and rheumatoid arthritis trials.

supplementation in RA underlined that the trials in which a significant reduction of CRP was achieved used the same probiotic strain, *L. casei* (Rudbane et al., 2018). Nevertheless, strain choice is only one of the various variables that are likely to influence the outcomes of probiotic studies, because dosage, study duration, frequency of administration, baseline disease activity (Marco and Tachon, 2013) and concomitant pharmacological treatment are also of major importance. **Table 2** details the characteristics and main findings of the included papers regarding probiotic effects on RA.

FERMENTED FOODS AS A POSSIBLE ALTERNATIVE TO PROBIOTIC SUPPLEMENTS

Fermented foods and beverages are defined as foods made through desired microbial growth and enzymatic conversions of food components (Marco et al., 2021). *Yoghurt, Kefir, Miso, Natto, Tempeh* and most *Kombuchas* are some of the available fermented foods that contain live microorganisms (Marco et al., 2021). Historically, many foods have undergone fermentation as a food preservation technique (Paul Ross et al., 2002; Dimidi et al., 2019) since the generation of antimicrobial metabolites like organic acids, ethanol and bacteriocins reduce the risk of contamination with pathogenic microorganisms (Dimidi et al., 2019). Nowadays, fermented foods and beverages are more popular than ever before (Bell et al., 2018). This emerging interest in such foods could be explained by their health-promoting potential (Marco et al., 2017; Melini et al., 2019).

Although the current body of evidence regarding the impact of fermented foods on health and disease remains insufficient (Bell et al., 2018; Dimidi et al., 2019), it is increasingly understood that some fermented foods promote human health in ways not directly attributable to the starting food materials (Marco et al., 2017). These foods could benefit health through the nutritive alteration of the ingredients, modulation of the immune system, or by influencing GM composition and activity (Marco et al., 2021). Most likely, several of these mechanisms are related and contribute to the effects of each other.

These foods contain various microbes with health-promoting properties, and GM has been suggested to be the mediator between fermented food consumption and these health outcomes. However, it is important to acknowledge that changes in the bacterial composition of diet do not necessarily translate into GM functional changes (Stiemsma et al., 2020). That being said, there is evidence from dietary interventions in humans suggesting that foodborne microbes can transiently colonize gut (David et al., 2014). Food ingested bacteria are capable of transient integration into GM, despite the resistance of resident communities to colonization by ingested bacteria (Derrien and van Hylckama Vlieg, 2015). Although these microorganisms are unlikely to maintain long-term residence in the intestine, it has been suggested that short-term colonization could be sufficient to synthesize bioactive compounds, inhibit intestinal pathogens and mediate epithelial modulatory effects (Marco et al., 2021). Nevertheless, to fully understand the long-term effects of the consumption of fermented foods, it is crucial that future studies have longer intervention periods and evaluate the GM composition, not only before and immediately after the

TABLE 2 | Probiotic effects on Rheumatoid Arthritis in clinical trials.

	Author/ year	Sample size/ study duration/ RA severity	Probiotic strain	Administration dose	Evaluated Parameters	Main findings
RCT with L. rhamnosus	(Hatakka et al., 2003)	*n* = 21/12 months/ mild disease	L. rhamnosus GG	≥5 × 10^9 CFU/capsule (4 caps/day)	HAQ; TJC and SJC; ESR; CRP; IL-1β; IL-6; IL-10; IL-12; TNF	No statistical differences were observed; ↑ number of subjects reporting subjective well being in the probiotic group
	(Pineda et al., 2011)	*n* = 29/3 months/ severity not described	L. rhamnosus GR-1L. reuteri RC-14	2 × 10^9 CFU/capsule (2 caps/day)	TJC and SJC; ESR; CRP; TNF; IL-1 α; IL-1β; IL-6; IL-10; IL-12; GH VAS; pain VAS; HAQ	No statistical differences were observed between groups
RCT with L.casei	(Vaghef-Mehrabany et al., 2014)	*n* = 46/8weeks/mild to moderate disease	L. casei 01	≥10^8 CFU/capsule (1 caps/day)	IL-1β; IL-6; IL-10; IL-12;TNF	↓ IL-6; ↓ IL-12; ↓ TNF;↑ IL-10
	(Alipour et al., 2014)	*n* = 46/8 weeks/mild to moderate disease	L. casei 01	≥10^8 CFU/capsule (1 caps/day)	DAS28; TJC and SJC; GH score; hs-CRP	↓ DAS28;↓ hs-CRP; ↓ GH score; ↓ TJC and SJC
	(Vaghef-Mehrabany et al., 2016)	*n* = 46/8 weeks/mild to moderate disease	L. casei 01	≥10^8 CFU/capsule (1 caps/day)	MDA; SOD; GPx; CAT; TAC	No significant effect was observed on the oxidative status
	(Zamani et al., 2016)	*n* = 60/8 weeks/ moderate to severe disease	L. acidophilusL. caseiB. bifidum	2x10^9 CFU/g of each strain (1 caps/day)	DAS28; TJC and SJC; VAS of pain; hs-CRP; serum insulin; HOMA-B; HOMA-IR; lipid profile; NO; TAC; GSH; MDA	↓ DAS28;↓ serum insulin; ↓ HOMA-B;↓ hs-PCR
	(Cannarella et al., 2021)	*n* = 47/60 days/ severity not described	L. casei LC-11L. acidophilus LA-14Lactococcus lactis LL-23B. Lactis BL-04B. Bifidum BB-06	10^9 CFU/sachet of each strain (1 sachet/day)	DAS28; GH VAS; TJC and SJC; WBC; ESR; hs-CRP; TNF; IL-6; IL-10; adiponectin; LOOH; PC; NO; SH	↓ WBC; ↓ TNF;↓ IL-6; ↓ NOx;↑ Total antioxidant capacity (TRAP); ↑ SH
RCT with *Bacillus coagulans*	(Mandel et al., 2010)	*n* = 45/60 days/ severity not described	Bacillus Coagulans GBI-30, 6086	2x10^9 CFU/day	HAQ-DI; TJC and SJC; ERS; CRP; pain score; global assessment	↓ pain score and CRP

B, bifidobacterium*; CAT, catalase; CRP, C-reactive protein; DAS28, disease activity score 28 joint count; ESR, erythrocyte sedimentation rate; GH, global health; GSH, glutathione; GPx, glutathione peroxidase; HAQ, Health Assessment Questionnaire; HAQ-DI, Stanford Health Assessment Questionnaire Disability Index; HOMA-B, homeostatic model assessment-B cell function; HOMA-IR, homeostasis model of assessment-estimated insulin resistance; hs-CRP, serum high sensitivity C-reactive protein; IL, interleukin; L.* lactobacillus*; LOOH, lipid hydroperoxide; MDA, malondialdehyde; NO, nitric oxide; NOx, nitric oxid metabolites; PC, protein carbonyl; RCT, randomized clinical trial; RA, rheumatoid arthritis; SH, sulfhydryl groups; SJC, swollen joint counts; SOD, superoxide dismutase; TAC, total antioxidant capacity; TJC, tender joint counts; TNF, tumour necrosis factor; TRAP, total radical-trapping antioxidant parameter; VAS, visual analogue scale; WBC, white blood cell counts.*

intervention, but also some time after the cessation of the regime (e.g., several weeks). *Bifidobacterium* and lactic acid bacteria, including *Lactobacillus*, which were studied as probiotic supplements in RA patients, are some of the microorganisms present in many fermented foods (Tamang et al., 2016).

Even though fermented foods can be classified as probiotics if they meet the required criteria, it is important to clarify that fermented foods are not equivalent to probiotic foods since many fermented foods do not have evidence of a demonstrated health benefit conferred by well-defined and characterized live microorganisms (Hill et al., 2014; Marco et al., 2021). The effects of the microorganisms are strain specific and most likely dose-dependent. This is one of the main limitations regarding the use of fermented foods as an alternative to probiotic supplementation. Additionally, some foods and beverages produced by fermentation do not contain live microorganisms, such as bread, beer, wine and distilled alcoholic beverages, due to their inactivation by heat or physical removal by filtration or other means (Rezac et al., 2018). As previously discussed, fermented foods could lead to beneficial outcomes by various mechanisms besides providing living microorganisms to the gastrointestinal tract. Hence, these foods could carry a positive functional role even in the absence of live microorganisms in the finished product, considering that microbes are capable of modifying food constituents, may produce vitamins or other bioactive molecules and inactivate anti-nutritional factors (Marco et al., 2017; Rezac et al., 2018). Since several other components in the food matrix may positively influence health and it is possible that fermented foods carry additional benefits when compared with probiotic supplementation by itself.

Considering all these findings, fermented foods can be probiotic carriers and may be a promising alternative to probiotic supplementation for RA patients. Fermented foods may change the amounts and types of beneficial bacteria that live in human gut (Stiemsma et al., 2020) and, considering the emerging evidence linking dysbiosis with autoimmunity mechanisms (Horta-Baas et al., 2017), this could be particularly interesting for RA patients. Moreover, there is evidence of the beneficial effect of fermented foods in reducing inflammatory biomarkers in studies conducted in healthy individuals (Burton et al., 2017; Pei et al., 2017). Of interest to the subject discussed in this paper, fermented foods have also been studied in other inflammatory conditions and, although more research is needed, results suggested that these foods can exert beneficial effects in conditions characterized by chronic inflammation. For instance, an RCT conducted in patients with inflammatory bowel disease found that *kefir* consumption significantly decreased ESR and CRP serum levels in patients with Crohn's disease and concluded that this intervention may improve both symptoms and quality of life of these patients (Yılmaz et al., 2019). Furthermore, in patients with type 2 diabetes, the consumption of a probiotic yogurt containing 3.7×10^6 CFU/g of both *L. acidophilus* (La5) and *B. lactic* (Bb12), significantly decreased TNF levels (Mohamadshahi et al., 2014). A significant decrease in TNF levels, as a result of yogurt consumption, was also found in a RCT promoted by Chen et al., conducted in obese women with nonalcoholic fatty liver disease and metabolic syndrome (Chen et al., 2019). In this study conventional yogurt was used and a significant decrease in serum lipopolysaccharide (LPS), a biomarker of gut permeability, was also found as well as changes in GM composition, namely a decrease in the abundance of the *Firmicutes* phylum and the taxa within it (Chen et al., 2019). In line with this, a recent systematic review and meta-analysis of RCTs regarding fermented foods and inflammation reported that fermented foods might have beneficial effects in subjects with an inflammatory disease background (SaeidiFard et al., 2020), as is the case of RA patients. Lastly, dietary interventions are among the nonpharmacological therapies proposed to minimize the consequences of the disease in patients with established RA (Küçükdeveci, 2019). Finally, it has already been suggested that fermented foods may be an appealing complement to a whole-dietary pattern, such as the Mediterranean Diet, since both fermented foods and the Mediterranean Diet have similar anti-inflammatory pathways and may potentiate each other, resulting in a promising combination for RA patients (Dourado et al., 2020). Altogether, this evidence highlights the need for well conducted intervention studies with fermented foods in RA patients.

CONCLUSION

The link between gut dysbiosis and RA has expanded the interest in investigating the modulation of the GM as a possible adjuvant therapy for disease prevention and treatment. The increasing evidence reporting the positive effects of probiotic bacteria in animal models of arthritis has been leveraging the desire to transfer these benefits into clinical practice. However, only a small number of studies addressed the role of probiotics in the management of RA on human subjects and, to the best of our knowledge, no human trial has investigated the role of probiotics in a preventive approach. Research in this field is still in need of high-quality studies with larger sample sizes and longer treatment durations to ascertain the exact benefit of this promising treatment for RA patients.

Probiotics supplementation in RA seems to have no clinically significant adverse effects, but further research is needed to get a solid basis concerning the most appropriate strains for RA patients. As of now, *L. casei* seems to be the strongest candidate, and its potential effect on GM and immune system could be further explored to achieve new insights on this promising therapy for RA patients.

Moreover, fermented foods may be a possible alternative to probiotic supplementation, as some of these foods and beverages are known to be probiotic carriers with potentially similar health benefits. As the current body of evidence investigating the impact of fermented foods on health and disease remains insufficient, its proposed benefits on the human GM should warrant future research consideration.

AUTHOR CONTRIBUTIONS

MF and SC collected and screened literature, made the selection of reviewed articles and drafted the article. ED defined the structure of the paper and conceptualized the figures. ED, CG and JF revised the draft and clarified concepts. MF and SC wrote the article in its final format. All authors read and approved the final format.

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frontiers in Pharmacology

REVIEW
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Searching for the one(s): Using Probiotics as Anthelmintic Treatments

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Helminths are a major health concern as over one billion people are infected worldwide and, despite the multiple efforts made, there is still no effective human vaccine against them. The most important drugs used nowadays to control helminth infections belong to the benzimidazoles, imidazothiazoles (levamisole) and macrocyclic lactones (avermectins and milbemycins) families. However, in the last 20 years, many publications have revealed increasing anthelmintic resistance in livestock which is both an economical and a potential health problem, even though very few have reported similar findings in human populations. To deal with this worrying limitation of anthelmintic drugs, alternative treatments based on plant extracts or probiotics have been developed. Probiotics are defined by the Food and Agriculture Organization as live microorganisms, which, when consumed in adequate amounts, confer a health benefit to the host. It has been proven that probiotic microbes have the ability to exert an immunomodulatory effect both at the mucosa and the systemic level. The immune response against gastrointestinal helminths is characterized as a type 2 response, with high IgE levels, increased numbers and/or activity of Th2 cells, type 2 innate lymphoid cells, eosinophils, basophils, mast cells, and alternatively activated macrophages. The oral administration of probiotics may contribute to controlling gastrointestinal helminth infections since it has been demonstrated that these microorganisms stimulate dendritic cells to elicit a type 2 or regulatory immune response, among other effects on the host immune system. Here we review the current knowledge about the use of probiotic bacteria as anthelmintic therapy or as a complement to traditional anthelmintic treatments. Considering all research papers reviewed, we may conclude that the effect generated by probiotics on helminth infection depends not only on the parasite species, their stage and localization but also on the administration scheme.

Keywords: probiotics, helminths, inflammation, type 2 immune response, regulatory immune response

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INTRODUCTION

Helminths have co-evolved with mammals and infect over one billion people worldwide, mostly in non-industrialized countries (Hotez et al., 2008). Generally, helminths have complex life cycles, involving different stages and hosts. Most species of parasitic helminths occupy more than a single niche in a human host during their life cycle and, in most cases, helminths produce chronic infections. Despite worldwide efforts, the development of vaccines providing long-term protection against helminths has been hampered by multiple life cycle stages, antigenic variation between them,

evasion mechanisms and immunomodulation strategies (Bobardt et al., 2020; Drurey et al., 2020). At present, only a few vaccines against helminths are commercially available with a high level of protection (>90%) and they are applied only to ruminants (Claerebout and Geldhof 2020). Therefore, a major approach to control helminth infections in livestock is periodic chemotherapy with anthelmintic drugs. However, uncomplete drug treatment schemes, high rates of post-treatments reinfections and the rise of anthelmintic resistance makes a dangerous combo for the increase of helminth infections among livestock with the consequent impact on human health (Hotez et al., 2008).

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al., 2014). These benefits include the prevention of health problems, such as diarrhea, irritable bowel syndrome, inflammatory bowel disease and allergic disorders such as atopic dermatitis (Islam 2016). The mechanisms of action of probiotics involve colonization and normalization of perturbed intestinal microbial communities, competitive exclusion of pathogens and bacteriocin production, modulation of enzymatic activities related to metabolization of a number of carcinogens and other toxic substances, and production of volatile fatty acids, which play a role in the maintenance of energy homeostasis and regulation of functionality in peripheral tissues (Bermudez-Brito et al., 2012; Plaza-Diaz et al., 2019). Probiotics also reinforce intestinal barrier function and mucin production and modulate the activity of gut-associated lymphoid tissue and the immune system (Plaza-Diaz et al., 2019). Likewise, probiotics may interfere with the physiology of parasites in the gut. Furthermore, their secreted products may have anthelmintic effects and can reduce the virulence of many parasites and for this reason probiotics may be an integral part of helminth parasite control strategies (Berrilli et al., 2012). Here we review the current knowledge about the use of probiotic bacteria as anthelmintic therapy or as a complement to traditional anthelmintic treatments.

IMMUNE RESPONSE AGAINST HELMINTHS

In general terms, the immune response in helminth infections is a type 2 immune response which is characterized by the production of interleukin (IL)-4, IL-5, IL-9, IL-10, and IL-13 (Allen and Maizels 2011). Adaptive immune cells including $CD4^+$ T helper 2 (Th2) cells and B cells innate immune cells such as basophils, eosinophils, mast cells and innate lymphoid cells (ILCs) are important sources of type 2 cytokines and are important effector cells (Gause et al., 2020).

The epithelial cell barrier not only represents the first line of defense against helminths but also provides signals to initiate type 2 immune response. As helminths invade epithelial barriers and migrate through tissues, they cause considerable damage. The cell death of host cells is associated with the release of damage-associated molecular patterns (DAMPs), which trigger signaling pathways that contribute to the initiation of the type 2 response. Tissue damage is sensed by mucosal epithelial cells which promote the secretion of alarmins like IL-25, thymic stromal lymphopoietin (TSLP), and IL-33 (Wiedemann and Voehringer 2020). These alarmins induce activation and differentiation of type 2 immune cells which then release several other cytokines like IL-4, IL-5, IL-9, and IL-13. Epithelial cells also express a set of cytokines that educate dendritic cells (DCs) in promoting adaptive Th2 cell immunity and activate ILC2, basophils, eosinophils and mast cells; epithelial cells can also express chemokines such as CCL17, CCL22 and eotaxins (CCL11, CCL24 and CCL26) recruiting DCs, eosinophils, basophils, mast cells, and $CD4^+$ T cells (Hammad and Lambrecht 2015). ILC2 are important in this type of response since they are inducer and effector cells and, like $CD4^+$ T lymphocytes, express the transcription factor GATA3 and secrete IL-5, IL-9, and IL-13 at the beginning of the infection (Klose and Artis 2016; Kouchkovsky et al., 2017; Gurram and Zhu 2019).

IL-4 and IL-13 induce the proliferation of goblet cells, which secrete mucus and resistin-like molecule-beta (RELMβ). IL-4 induces IgE production; then, IgE-antigen immune complexes bind to high-affinity IgE receptors (FcεRI) on basophils and mast cells, causing the degranulation and release of several proinflammatory mediators, such as histamine, heparin and serotonin (Tantisira et al., 2007; Kubo 2018). IL-5 is responsible for the activation and recruitment of eosinophils, and IL-9 causes mast cell activation. Altogether, immune cells and the secreted cytokines coordinate parasite expulsion by increasing fluid and mucus production, encapsulation and barrier formation, epithelial cell turnover, smooth muscle cell contraction, and production of anthelmintic effector molecules, such as RELM-β (Babu and Nutman 2019). The type 2 cytokines mentioned also stimulate repair of tissues damaged by parasites, which control inflammatory processes and promote tissue remodeling and restructuring (Faz-López et al., 2016; Shapouri-Moghaddam et al., 2018).

Helminths have been shown to modulate/regulate the host response to their own benefit (parasite-specific immunoregulation) by releasing immunomodulatory molecules that evoke a regulatory phenotype among innate and adaptive immune cells (McSorley and Maizels 2012; Navarro et al., 2016; Wu et al., 2017; Gazzinelli-Guimaraes et al., 2018; Ryan et al., 2020). For this reason, and in the hygiene hypothesis context, helminth-derived products have been tested for treating autoimmune diseases (Yazdanbakhsh et al., 2002; R. M.; Maizels et al., 2014; Stiemsma et al., 2015). However, chronic helminth infections may induce allergic diseases as they enhance type 2 inflammation (Herz et al., 2000; Hurst et al., 2001; Demirci et al., 2003; Maizels and Yazdanbakhsh 2003).

Although helminth parasites are universal in inducing all or most of these type 2 effector pathways, in the host the specific effector pathway mediating protection varies between different parasites, lifecycle stages, and site of infection.

MECHANISM OF ACTION OF PROBIOTICS

To discuss the role of probiotics in parasite infections it is necessary to describe their general effects on the gut environment and their immunomodulatory capacity.

As mentioned, probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. Among these probiotics, one of the more important groups is that of lactic acid bacteria. These microorganisms have the ability to produce large quantities of lactic acid, which inhibits the growth of pathogenic bacteria, and have been used to produce dairy foods for centuries (Metchnikoff 1908; Mackowiak 2013; Fisberg and Machado 2015). The former genus *Lactobacillus*, subdivided into 25 genera (including 23 new ones), includes different species with proven probiotic activity, such as *Lacticaseibacillus casei*, *Lacticaseibacillus rhamnosus*, and *Lactobacillus delbrueckii* (Zheng et al., 2020). Moreover, other genera like *Bifidobacterium*, *Enterococcus* and *Streptococcus,* also include strains with probiotic capacity.

The ability to promote health benefits by these bacteria resides in direct effects on other microorganisms in the gut lumen as well as in the modulation of immune and non-immune cells (Plaza-Diaz et al., 2019). Regarding the direct effects, besides the production of lactic acid, these bacteria can proliferate in the gut, preventing the colonization by different pathogens. Moreover, probiotics produce and secrete different bacteriocins, peptides that affect the growth of bacteria, fungi, parasites, and viruses (Hernández-González et al., 2021). They also produce short-chain fatty acids (SCFA), like butyrate and acetate, and branched-chain fatty acids (BCFA), such as isobutyrate and 2-methylbutyrate. These volatile fatty acids help the host cells, including immune ones, to maintain energy homeostasis.

Regarding the effects produced specifically on the immune system, they are triggered by interactions between the microorganisms and cellular receptors. The latter are present both in epithelial and immune cells, being the dendritic cells (DC) that emit cytoplasmic processes into the gut, an essential mediator of probiotics' effects (Sánchez et al., 2017). The bacterial surface molecules recognizable through specific receptors include the peptidoglycan, lipoteichoic and teichoic acids (surface molecule of gram-positive bacteria), surface proteins, and different glycan residues present in surface molecules. These molecular targets are recognized by receptors such as toll-like receptor 2 (TLR2), TLR6, nucleotide-binding oligomerization domain-containing protein 1 (NOD1), NOD2, and others. The receptor-target interaction mediates effector responses that usually depend on the gut environment. Under physiological conditions, these responses tend to promote immune tolerance, a lack of specific effector inflammatory responses, and the avoidance of damage to the microorganisms and the surrounding tissue. However, activated DC can also prime T cells, inducing effector Th1, Th2, or Th17 responses against different targets, such as microorganisms and cancer cells (both local and distant ones) (de Moreno de LeBlanc et al., 2007). Probiotics can also modify immune responses through the secretion of different molecules. In this way, the role of lactic acid production on gut immunity has been described. *In vitro* assays showed that lactate decreases inflammatory responses of DC and macrophages, an effect that correlates with reduced intestinal inflammation in a murine colitis model (Iraporda et al., 2015; Iraporda et al., 2016). Besides lactic acid, SCFA also affect immune cell metabolism and the overall immune response (Tan et al., 2014). However, probiotics can also modulate immune cells in an indirect way. When enterocytes recognize molecular targets on probiotics, these cells are capable of secreting a wide variety of pro- and anti-inflammatory cytokines which ultimately affect immune cells (Corthésy et al., 2007). The effects mentioned do not require live probiotics to be produced. It has also been observed that heat-killed bacteria as well as molecules isolated from probiotics, such as lipoteichoic acid, can exert some of the effects observed for live microorganisms (Friedrich et al., 2017).

The local effects produced by probiotics on the gut environment and the gut-associated lymphoid tissues (GALT) have allowed their use as a treatment or as a supplementary therapy on different gastrointestinal diseases. Such uses go from treating gut dysbiosis (produced by antibiotic therapy, diarrhea, food intake, or others) to restoring the microbial balance in the gut (McFarland 2014) or modulating the immune response in inflammatory diseases and cancer. Briefly, probiotics' use in inflammatory bowel diseases, being ulcerative colitis (Dang et al., 2020) and Crohn's disease (Lichtenstein et al., 2016) the most frequent pathologies, has shown different grades of efficacy (Orel and Trop 2014). Whereas there was some efficacy in regulating inflammation in ulcerative colitis, there was no evidence of significant beneficial effect in Crohn's disease. However, the immune system modulation induced by probiotics is beneficial in the case of gastrointestinal tumors. In this case, probiotics exert a prophylactic effect (observed both in animal models and in epidemiological studies) and protect the gut against side effects of antitumor treatments (Drago 2019). Some of the modulatory effects are related to the cytokines induced in the probiotic-treated animals, including TNF-α, IFN-γ, and IL-10 (de LeBlanc and Perdigón 2004).

Nevertheless, the immune modulation induced by oral probiotics is not limited to the gastrointestinal tract. These effects can impact on distant organs, such as the lungs and the skin (Friedrich et al., 2017). This can be observed in atopic diseases, such as asthma and atopic dermatitis (AD). Regarding the respiratory atopic disease, an interesting meta-analysis showed that probiotics' administration reduced the number of episodes in treated children, with a concomitant reduction in IL-4 production and an increase in IFN-γ levels, showing a bias of the T helper response towards a Th1 profile. However, no statistical differences were found in other outcomes of the pathology (Lin et al., 2018). According to these findings, a meta-analysis done by Wei and colleagues showed that the use of probiotic supplementation did not associate with a lower risk of asthma in infants (Wei et al., 2020). In the case of AD, a significant number of clinical trials revealed AD prevention with probiotics' consumption in children (Meneghin et al., 2012). Moreover, the authors also reported the therapeutic effectiveness of the treatment with different beneficial microorganisms once the pathology was installed (Meneghin et al., 2012). In a more recent meta-analysis, Rusu and colleagues argue that despite these results there is not enough data regarding optimal dosing, optimal time to start treatment and duration necessary to show beneficial effects (Rusu et al., 2019).

Finally, the immunomodulatory capacity of probiotics was also demonstrated in skin cancer studies. *Lactobacillus* spp. lipoteichoic acid produced a reduction in squamous cell carcinomas in chronically UV-exposed mice, reinforcing Th1 response (Weill et al., 2013). Moreover, this treatment was also effective in preventing UV-induced immunosuppression (Friedrich et al., 2019). In addition, the oral treatment with *Bifidobacterium* spp. was as effective as PDL-1 blocking monoclonal antibody in affecting melanoma's growth in a mouse model (Sivan et al., 2015).

Overall, probiotics affect the balance of gut microbiota and modify the availability of molecular targets for immune receptors and soluble metabolites, leading to modulation of innate and adaptive immune responses. These modulations are induced both at the local and the systemic levels (Sivan et al., 2015).

PROBIOTICS AS COMPLEMENT/ TREATMENT AGAINST HELMINTH INFECTIONS

Experimental evidence on the use of probiotics to treat parasite infections is limited. It is important to highlight the lack of blind, placebo controlled, clinical trials. Consequently, most of the published work commented hereafter was developed in different animal models, including experimental models (typically mouse) and susceptible animals (such as pigs).

Evidence about the role of gastrointestinal bacteria on parasite infection can be found in the correlation between microbiota composition and parasite infection. Reynolds et al. reviewed the effects of the presence of parasites on the microbiota, and vice versa (Reynolds et al., 2015). Interestingly, the absence of microbiota (in germ-free mice) hampers parasite infection. Moreover, the presence or administration of some *Lactobacillus* strains promote helminths colonization or persistence. The authors highlight that *Lactobacillus* spp. tend to decrease type 2 responses and increase Tregs, as mentioned, and propose that those mechanisms may explain the results observed in germ-free mice.

Here, we present experimental and epidemiological evidence about the role of probiotics and commensal bacteria on different helminth infections.

Nematodes

Ascaris spp.

Hosts contract *Ascaris* spp. infection *via* the faecal-oral route; when embryonated eggs are ingested, larvae penetrate the gastrointestinal tract and enter the blood stream. Through the blood stream the larvae are carried to the liver and heart, then enter pulmonary circulation and are released in the alveolar space, where they grow and molt. From the respiratory system the larvae are coughed up, swallowed, to finally reach the small intestine, where they mature to adult male or female worms. After mating, female worms release eggs that contaminate faeces.

Different works were published studying the effect of probiotics during this helminth infection, including some in pigs infected with *Ascaris suum* (Jang et al., 2017; Solano-Aguilar et al., 2018). The first report, by Jang et al., described the use of *L. rhamnosus* GG (LGG) as a probiotic treatment of the infection. Moreover, the authors included flavonol-rich cocoa powder (CP) as a supplement, due to the immunomodulatory capacity of the flavonols such as catechin and epicatechin present in the extract. The treatment was applied during 5 weeks prior to parasite challenge, and the animals were evaluated 17 days after inoculation. No significant differences in intestinal parasite content (L4 larvae) were observed, but a delay in intestinal expulsion of parasitic larvae from the intestine was registered in the CP + LGG group. No changes in serum specific IgG2 were obtained with the probiotic treatment. Interestingly, the LGG treatment alone induced modifications of cytokine and chemokine transcription (decrease of IL-1β, IL-13, and CCL26) in the tracheobronchial lymph nodes but not in the mesenteric lymph nodes (Jang et al., 2017).

Using the same model of host and parasite, Solano-Aguilar et al. evaluated the effect of *Bifidobacterium animalis* subspecies *lactis* with the modulation of the intestinal immune response. The probiotic treatment began on the sows and continued in newborn piglets for 2.5 months, when they were inoculated with *A. suum*. Even though they did not observe changes in the number of intestinal L4 larvae at 17 days post infection (p.i.), an increase in specific antibodies was detected (serum IgA and ileum fluid IgG1 and IgG2). Moreover, jejunal mucosa from infected pigs showed a characteristic decrease in glucose absorption and an increase in the secretory response to histamine, both being attenuated by the probiotic treatment. Finally, the eosinophilia promoted by the parasite was decreased by *B. animalis* subspecies *lactis*, without affecting the expulsion of the worms (Solano-Aguilar et al., 2018).

In another work, the therapeutic ability of inactivated *Bacillus thuringiensis* overexpressing Cry5B protein (paraprobiotic with anthelmintic properties) was tested in *A. suum* infection models. The paraprobiotic intoxicated *A. suum* larvae *in vitro* and was highly effective against intestinal *A. suum* infections in a mouse model. In pigs, a single oral dose of this paraprobiotic reduced the parasite burden by 96% during *A. suum* infections (Urban et al., 2021). This strategy results in an attractive alternative for the control other helminth infections.

All these data suggest that during *Ascaris* spp. infection depending on the treatment realized with probiotics, the effects on parasitological and immunological parameters could be beneficial, negative, or neutral. The scarcity of studies on the effects of probiotics against this helminth infection clearly indicates the need of further research on this topic.

Trichuris spp.

Trichuris trichiura is one of the most common gastrointestinal nematodes. In infected children, trichuriasis is associated with malnutrition, growth stunting, and reduced educational performance, whereas in adults, it is related to anemia, reduced worker productivity, and/or low-birth-weight babies (Bethony et al., 2006). The cycle initiates by ingestion of contaminated water or food with embryonated eggs, which hatch in the intestine to release infective L1 larvae. L1 migrate to the caecum and colon and undergo four molts to adult worms. Adult females produce eggs, which are excreted in the feces.

Infective eggs in the environment are subsequently ingested. *Trichuris muris* in the mouse has provided a useful and relevant model system with which to explore immunity to *T. trichiura* in man due to their homology at the genomic and transcriptomic level (Klementowicz et al., 2012; Foth et al., 2014). Infection of mice with *T. muris* drives polarized T helper cell (Th) responses, which associate with resistance (Th2) or susceptibility (Th1) (Klementowicz et al., 2012). B cells are important in the development and maintenance of the protective immune response to *T. muris* (Sahputra et al., 2019).

The role of probiotics inducing regulatory immune responses was discussed above. However, implications of this mechanism against parasite infection are not necessarily straightforward. McClemens et al. showed the effects of *L. rhamnosus* (JB-1) administration against *Trichuris* spp. infection, highlighting the role of IL-10 on those effects. Susceptible and resistant mouse strains (AKR and C57BL/6, respectively) were inoculated with *T. muris* and fed with live and γ-irradiated probiotics. The procedure was conducted also in IL-10 KO mice. The number of worms in the caecum was reduced by live *L. rhamnosus* at 10, 14, and 21 days post-inoculation (*vs.* control), but the effect was lost when dead bacteria were used. The increased parasite expulsion induced by this probiotic was also lost in IL-10 KO mice, showing the important role of this regulatory cytokine. Moreover, the AKR mouse strain, susceptible to develop chronic infections with *T. muris*, also showed a decrease in parasite burden after *L. rhamnosus* treatment. An increase in IL-10 production was observed in these mice, reinforcing the results about the role of this cytokine in the expulsion of the nematode (McClemens et al., 2013).

In another work, Dea-Ayuela et al. studied the effect of an oral treatment with either viable or dead *L. casei* (ATCC 7469) before *T. muris* infection. The results in the treated groups were not encouraging; both conditions favored the infection as the mean number of L3 larvae recorded were significantly higher than in challenged untreated controls. Regarding the immune response, viable *L. casei* reduced the levels of fecal IgA induced by challenge infection, decreased the cellular response (diminished proliferation of MLN cells with mitogens), and reduced IFN-γ, TNF-α, IL-4 and IL-13 in both MLN and PP compared to infected untreated mice (Dea-Ayuela et al., 2008).

When we compare both studies, results seem to depend on the species of probiotic used. While *L. rhamnosus* treatment had a protective effect on *T. muris* infection, critically dependent on IL-10, *L. casei* treatment diminished the immune response at both the cellular and humoral level, leading to a higher parasite load.

Trichinella spiralis

The life cycle of *T. spiralis* is completed in one host and there is no free-living stage. Transmission occurs when infected skeletal muscle containing muscle larvae (ML or L1) is consumed. In the stomach, ML are freed from muscle by digestion and move into the small intestine where they invade epithelial cells and migrate through the epithelial monolayer creating syncytia. ML molt four times becoming adult worms (AW) who mate in the epithelium. Female worms release newborn larvae (NBL) which enter the lymphatics and eventually reach the bloodstream. During their journey to their final ecotope, NBL can extravasate in any tissue, such as liver, lungs, or heart, but can only complete their development in skeletal muscle cells. When fully mature, each larva can infect a new host.

Clearance of AW from intestine is mediated by a potent type 2 response, which is characterized by increases in the numbers of lymphocytes, eosinophils, goblet cells and mast cells, and also by the switch to IgE and IgG1 isotypes (Ahmad and Bell 1991; Negrão-Corrêa 2001; Saracino et al., 2020). Regarding the NBL, it is known that they are killed by antibody-dependent cellular cytotoxicity (ADCC) both at systemic and tissue levels (Kazura and Grove 1978; Wang and Bell 1987; Venturiello et al., 1993; Gentilini et al., 2011; Falduto et al., 2014).

Bautista-Garfias et al. studied the ability of viable *L. casei* (ATCC 7469), administered by an intraperitoneal route, to induce resistance in mice against *T. spiralis* infection. The percent of reduction of adult worms in the intestine 5 days after *T. spiralis* infection observed in *L. casei*-treated animals, compared with those of the control group, fluctuated between 70.9 and 88.5%; and the reductions of larvae per gram of muscle tissue, evaluated at 30 days after infection, varied from 46.6 to 84.4% (Bautista-Garfias et al., 1999). Later, this group evaluated the effects of *L. casei* (ATCC 7469 live, heat-killed and culture supernatant) orally administered prior to oral challenge with *T. spiralis*. The treatment with live probiotic reduced AW numbers by 58%, whereas dead probiotic did it by 44% and culture supernatant by 32%. ML were also decreased by 70, 65 and 24% by live bacteria, dead bacteria, and culture supernatant, respectively (Bautista-Garfias et al., 2001). These results show that, in the response against *T. spiralis*, the immune mechanisms triggered by probiotics components are enough to induce the observed effects. Moreover, soluble mediators produced by *L. casei* are also effective, even though with a lower potency. Comparing administration routes, intraperitoneal versus oral, it seems that the parasitic load reductions were higher with administration by intraperitoneal route.

A more recent study of the group analyzed the effect of the intraperitoneal administration of *Lacticaseibacillus casei* Shirota strain in CD1 mice on the establishment of *T spiralis* AW, and on the generation of intestinal IgA anti-*T. spiralis* after challenge. From day 5 p.i., mice in *L. casei* Shirota group showed a significantly smaller number of AW and higher levels of IgA anti-*T. spiralis* than animals from the untreated group which suggest that *L. casei* Shirota would be protecting mice from *T. spiralis* infection (Martínez-Gómez et al., 2009).

More recently, an interesting approach to evaluate the role of immune modulation and probiotics in *T. spiralis* infection was published. Wang et al. treated mice with both wild type and IL-4 recombinant *Lactiplantibacillus plantarum* NC8 prior to challenge with *T. spiralis*. A marked reduction of the infection-induced weight loss was observed with both treatments. However, wild type *L. plantarum* failed to reduce intestinal adult worms at day 7 post-infection, whereas the IL-4-expressing bacteria produced a significant decrease. Interestingly, the number of ML found at day 28 after parasite inoculation was significantly reduced by both treatments (being the effect of the recombinant bacteria more intense) (Wang et al., 2020). This work shows that *Lacticaseibacillus* spp. administration can partially reduce

parasite burden, and that the effect is stronger in the presence of IL-4. However, these results contrast with those obtained by Temsahy et al. who found that serum IFN-γ concentration was raised in mice fed with *L. plantarum* P164 whether they were or not infected with *T. spiralis* (Temsahy et al., 2015). Actually, the authors attribute this IFN-γ increase to the bacterial peptidoglycan and not, as other authors, to the infection by *T. spiralis* (Dvorožňáková et al., 2011; Gentilini et al., 2011) or the combination of both stimuli. These authors also showed that the probiotic was able to colonize the gut after probiotic feeding, which they explained as a result of its adhesion ability.

Dvorožnáková et al. explored the effects of *Enterococcus faecium* CCM8558, *Enterococcus durans* ED26E/7, *Limosilactobacillus fermentum* CCM7421 and *L. plantarum* 17L/1 on cellular immunity. Mice treated with probiotic strains and then infected with *T. spiralis* showed a higher cellular response in terms of phagocytosis and respiratory burst (Dvorožňáková et al., 2016). Moreover, when the distribution of $CD4^+$ and $CD8^+$ cells was studied in the intestine they found that there was a higher number of $CD8^+$ cells at the epithelia and increased numbers of $CD4^+$ cells at lamina propria, which could contribute to the reduction in the number of adult worms in the host (Dvoroznakova et al., 2016). Regarding this point, Temsahy et al. found an increase in goblet cells hyperplasia in the intestines of mice treated with *L. plantarum* which could also explain the lower number of AW recovered from the gut as these cells are involved in AW expulsion (Temsahy et al., 2015).

Bucková et al. have shown that the parasite burden, the number of adult worms, the female fecundity and NBL were diminished when mice were treated with *E. faecium* CCM8558, *E. durans* ED26E/7 and *L. fermentum* CCM7421. Also, these probiotic strains reduced the female fecundity with the subsequent reduction in the number of NBL in the *in vitro* assays (Bucková et al., 2018).

Toxocara spp.

Humans are accidental hosts of *Toxocara* spp. who become infected by ingesting infective eggs or undercooked meat/viscera of infected hosts. After ingestion, the eggs hatch, and larvae that penetrate the intestinal wall are carried by the circulation to a variety of tissues (liver, heart, lungs, brain, muscle, eyes). While the larvae do not undergo any further development in these sites, they can cause local reactions and mechanical damage leading to clinical toxocariasis. *T. canis* causes larva migrans syndrome that induces an immune response characterized by blood eosinophilia, eosinophilic infiltration around larval sites of migration, specific antibody production (IgG and IgE) and a Th2 response (Ruiz-Manzano et al., 2019). Migrating larvae are attacked by host immune responses, resulting in local inflammation associated with eosinophilia and increased production of cytokines and specific antibodies. Although many *T. canis* infections are subclinical in nature, human toxocariasis can manifest itself as syndromes known as visceral larva migrans, ocular larva migrans, neurotoxocariasis, and covert or common toxocariasis (Taylor et al., 1988; Finsterer and Auer 2007; Pivetti-Pezzi 2009).

Regarding *Toxocara* spp. infection and probiotic treatment, several experimental reports are published. Most of them evaluated the parasite burden after pretreatment with probiotics in mouse models. Moreover, the direct effect of the microorganisms on parasite viability was evaluated *in vitro* in many of these works. *Saccharomyces boulardii* is a non-bacterial microorganism, closely related with *S. cerevisiae*, with probiotic properties which also is resistant to the adverse conditions of gastric and intestinal environments (Pais et al., 2020).

de Avila et al. (2012) showed that *S. boulardii* was able to reduce the number of larvae in Swiss mice infected with *T. canis* at both the acute and chronic phase of the infection. However, *in vitro* assays did not show a larvicidal effect on L3 larvae (de Avila et al., 2013) suggesting the necessity of a contact of *S. boulardii* with the intestinal mucosal or microbiota to mediate the observed *in vivo* effects. In a subsequent, the authors explored the effect of *S. boulardii* on the immune system in mice infected with *T. canis* (de Avila et al., 2016). The cytokine secretion in splenocytes from mice orally treated or not with *S. boulardii*, and later infected with *T. canis*, was evaluated. The study showed that diet supplementation with *S. boulardii* stimulates a Th1 response since IL-12 and IFN-γ genes transcription was elevated in both infected and not infected groups whereas IL-4 and IL-10 did not present any significant differences between treatments. In a later work, this group used a different infection model as mice were infected with larvae and not with *T. canis* eggs. This study not only did not reveal a significative reduction of *T. canis* larvae, but also showed that IL-12 transcription was below the threshold value in both supplemented and not supplemented infected mice. It is worth to notice that uninfected animals supplemented with the probiotic showed a significant increase in this cytokine transcription in the duodenum (de Moura et al., 2017). Taken together these results point out that *S. boulardii* anthelmintic properties will depend on the parasitic stage that is aimed to eliminate. Finally, these researchers studied the effect of *S. boulardii* when animals are infected by the ingestion of raw liver from chickens infected with *T. canis* larvae in order to emulate the natural infection. Mice that received diet supplemented with *S. boulardii* showed a reduction of 64.4% in the number of larvae recovered from the liver and 66.7% from the lungs as compared to those not treated with the probiotic.

The effects of *L. rhamnosus* (ATCC 7469) and *L. acidophilus* (ATCC 4356) on the experimental infection with *T. canis* were also investigated. The probiotic treatment was initiated before parasite challenge with *T. canis* embryonated eggs. These probiotics successfully reduced the number of migrating larvae found in liver at 48 h p.i. (52% reduction for *L. rhamnosus* and 58% for *L. acidophilus*). As previously found for *S. boulardii*, *L. rhamnosus* and *L. acidophilus* displayed their effects *in vivo* but did not exert a direct effect on the parasite *in vitro*, demonstrating once more that the interaction between the host and these bacteria is of paramount importance for the protective effects (de Avila et al., 2012; Walcher et al., 2018; Cadore et al., 2021).

Another probiotic evaluated regarding the prevention of *T. canis* infection was *E. faecalis* (CECT 7121). In this case, co-administration of probiotic and parasite led to a reduced number of larvae recovered from liver and lungs (Basualdo et al., 2007).

However, if the parasite infection was done before probiotic treatment the effect was lost (Chiodo et al., 2010).

Trematodes

Schistosoma spp.

During infection with *Schistosoma* spp. adult worms produce eggs which exit the host *via* urine or fecal matter. When schistosome eggs enter freely into the environment they hatch to produce miracidia that invade snails and develop along different stages. Cercariae travel from the intermediate snail host to the definitive mammalian host. Following attachment and skin penetration, cercariae transform to schistosomula which travel to the lungs, and then to the liver. Within the liver, worms develop into female and male adults that finally lodge within the portal and mesenteric vessels of the small intestine or the veins of the vesical and pelvic plexuses, from where the gravid females release eggs. Unfortunately, many bloodborne eggs become lodged within vascularized tissues and organs being the main cause of pathology following infection (Burke et al., 2009). The pathogenesis of schistosome infections and the morbidity associated with infection is due to a lethal combination of highly immunogenic eggs, a vigorous immune response and the various organs in which eggs become trapped.

To restrain the invading cercariae, innate and local stromal cells trigger an inflammatory cascade, with the release of macrophage inflammatory protein (MIP)-1, IL-6, IL-1, IL-12/23p40 and IL-18. Cercarial products can also directly stimulate production of cytokines, such as IL-4 and IL-10, which dampen the Th1 inflammatory response via their antagonistic effects on IL-12/23p40 production.

In the first weeks of murine *S. mansoni* infection, while the host is exposed to schistosomula and semi-mature schistosomes, a Th1-like immune response is observed. With the onset of egg deposition, however, a pronounced Th2 immune response comes up being characterized by high production of IL-4 and IL-13, IgE synthesis as well as eosinophilia and mastocytosis. Finally, when infection becomes chronic and egg production continues, a general down-modulation of immune reactivity develops, leading to a diminished Th2 response together with a smaller size of newly formed granulomas (Pearce and MacDonald 2002). The eggs induce a granulomatous host immune response largely characterized by lymphocytes (which mainly produce Th2 cytokines), eosinophils, and alternatively activated macrophages (Pearce and MacDonald 2002; Fairfax et al., 2012). This response eventually sequesters egg products, but it can also lead to severe hepatic fibrosis and portal hypertension.

In the case of schistosomiasis, the effectiveness of probiotic treatment was studied alone or combined with antiparasitic drugs. Zowail et al., 2012 evaluated the treatment of mice previously infected with *S. mansoni* with either *Bacillus coagulans*, praziquantel (drug of choice for the control of schistosomiasis) or a combination of both. They observed a 53% reduction in the number of adult worms using the probiotic alone, an 89% with the standard praziquantel treatment, and a 100% reduction with the combined treatment. Moreover, the number of eggs in the liver and intestine was also reduced in a similar way; for the liver, reductions of 47, 59 and 87% were observed, whereas for the intestine the reductions were 51, 53 and 71% (probiotic alone, praziquantel, and combined therapy, respectively). This work highlights the importance of evaluating the probiotics as a supplement for regular antiparasitic treatments (Mohamed et al., 2016). As other authors have demonstrated, infection by *S. mansoni* induces chromosomal aberrations and DNA damage (Shubber and Salih 1987) as well as praziquantel treatment (Montero and Ostrosky 1997) so in order to prevent this effect, El-Esawy et al. used *B. coagulans* as a complement of anti-parasitic treatment. When anthelmintic treatment was combined with *B. coagulans* a significant reduction in chromosomal aberrations induced by infection or praziquantel treatment was observed. This work highlights the importance of evaluating the probiotics as a supplement for regular antiparasitic treatments (El-Esawy 2012).

In a more recent work performed by El-Khadragy et al., the efficacy of a mixture of *L. acidophilus* (ATCC 4356) and *L. delbrueckii* subsp. bulgaricus (DSM 20080) was evaluated in a mouse model of *S. mansoni* infection. In this work, probiotics were administered either before or after parasite challenge. Moreover, the probiotic mix was applied in saline buffers and as a yogurt (allowing the probiotic to ferment milk for 5 h). Although results were also compared with praziquantel treatment, combined therapy was not evaluated. The authors observed a reduction in adult worm burden in mice treated with either probiotics or yogurt, both in administrations pre- and post-infection (68 and 60% for probiotics pre- and post-infection, and 72 and 64% for yogurt pre- and post-infection). Probiotics were almost as effective as praziquantel, which produced a 78% decrease in the number of adult worms. The reduction in liver eggs followed a similar pattern. Interestingly, *S. mansoni* infection increased the hepatic levels of MMP-9, lipid peroxidation, and NO, and decreased the levels of reduced glutathione. All these effects were prevented or reverted by probiotic treatment, as well as by praziquantel. Interestingly, the drug failed to decrease hepatic NO levels, whereas all probiotic treatments were successful (El-Khadragy et al., 2019).

De Fátima Macedo Santos et al. studied the effect of *Zymomonas mobilis* administration in mice divided into prophylactic and curative groups. Also, a mixed group was considered which received bacterial culture before and after *S. mansoni* infection. The total number of adult worms recovered was lower in the curative group, resulting in 61% protection. However, when prophylactic treatment was applied there was a non-significant reduction of adult worms. Surprisingly, animals belonging to the mixed group had an exacerbation of the infection, with a larger number of adult worms (De Fátima Macedo Santos et al., 2004).

Ghanem et al. used yoghurt containing *L. casei*, *L. acidophilus*, *L. plantarum* and *Limosilactobacillus reuteri* to fed mice before and after infection with *S. mansoni*. This strategy resulted in body weight gain as well as decreased spleen and liver weights to values close to controls. This probiotic yoghurt was found to display an immunomodulatory effect by stimulating an IgM response against soluble worm antigens as compared to the untreated control. While the infection increased AST, LDH and gGT activity in plasma, the addition of probiotic yoghurt led to a significant decrease of these enzymes in infected animals (Ghanem et al.,2005).

Taken together, these studies suggest that probiotics treatment may be used before or after infection with *Schistosoma* spp. However, as suggested by the study of De Fátima Macedo Santos et al., the combination of both treatments should be studied previously in each model to rule out potential adverse events such as increased parasitic load. This exacerbated infection may be due to an anergy state.

Cestodes

Echinococcus spp.

The two species with clinical importance are *E. granulosus* and *E. multilocularis*. The life cycle of this cestode involves dogs and other canids as definitive hosts, and sheep and other herbivores as intermediate hosts. After ingestion by a suitable intermediate host eggs hatch in the small intestine and release an oncosphere that actively penetrates the intestinal wall and migrates through the circulatory and lymphatic system into several organs, particularly the liver and lungs. In these organs, the oncosphere develops into a metacestode (cyst) that gradually enlarges, producing protoscolices and daughter cysts that remain within the cyst. The definitive host is infected after ingesting the cyst-containing organs of the infected intermediate host. After ingestion, the protoscolices evaginate, attach to the intestinal mucosa and develop into adults.

Humans are accidental intermediate hosts and become infected via ingestion of eggs which hatch and develop into metacestodes (fluid-filled cysts, hydatids) in tissues, particularly the liver and lungs. Two essential mechanisms appear to be at the basis of the often long-lasting and asymptomatic co-habitation of the hydatid cyst and the intermediate host: immune evasion/modulation and protective immunity to re-infection. The latter is antibody- and complement-dependent (Dempster et al., 1992; Dempster et al.,1995; Heath and Lawrence 1996; Lightowlers 2010; Torben et al., 2012), and is enhanced in the presence of neutrophils (Rogan et al., 1992). A mixed Th1/Th2 response, together with high levels of IL-10, is evoked as shown by *ex vivo* stimulation of splenocytes with protoscoleces (PSC) extract. The production of IL-10 and IL-4 could be actively induced by the parasite to favor its establishment (Dematteis et al., 1999).

Regarding the effects of probiotics in echinococcosis, there are few but interesting studies. Yousif and Ali studied the effect of a mix of *L. acidophilus*, *L. casei* and *L. rhamnosus* in the immune response against infection with secondary hydatid disease as an antiparasitic immunomodulator in BALB/c mice. The bacteria were administered by intraperitoneal route in mice, before and after infections with *E. granulosus* protoscoleces. Many criteria were considered, including numbers, weight, diameter, and percentage reduction of hydatid cysts of treated mice as compared to infected animals not treated with probiotics. The study showed a decline in cysts, including their diameter, weight, and number in probiotic treated animals. The bacteria were applied at two different concentrations, both promoting the reduction in number, size and diameter of hydatid cysts 6 months post-infection (98.03% reduction). It may well be concluded that probiotic bacteria can be used as a therapeutic method against hydatidosis. Unfortunately, no immune analysis was done (Yousif and Ali 2020).

Vogt et al. presented a different approach: they developed and used a recombinant *Bacillus subtilis* strain, carrying two antigens from *E. granulosus*. This approach is based on the capacity of the recombinant bacteria to act as a delivery system for the vaccination antigens. The work was done in dogs and the response evaluated was the production of specific antibodies in the serum of the treated animals. Dogs generated a humoral response, mainly IgG, not only against E. granulosus peptides but also against some B. subtilis antigens (Vogt et al., 2018). However, it is interesting the use of a well-known and safe probiotic microorganism as delivery system, which may have an impact *per se* against a challenge with the parasite.

Other Helminths

Hookworms

Hookworms are soil-transmitted nematode parasites that can reside for many years in the small intestine of their human hosts, where they suck blood and can cause iron deficiency anemia (Loukas et al., 2016). Two major species of hookworms infect humans: *Necator americanus* and *Ancylostoma duodenale*. During the life cycle of the hookworm eggs expelled in the feces of the infected host hatch in the environment, resulting in L1 larvae, which then molt twice to L3 (infective). L3 penetrate the skin of mammalian hosts. The larvae then enter the bloodstream, migrate through heart to the lungs, break through the alveoli, creep up the trachea and are swallowed, eventually residing in the small intestine to mature to adult worms. In the small intestine, adult hookworm mate and produce eggs that are passed in the feces, completing the life cycle.

Human hookworm infection generates a robust specific Th2 response, with some evidence of a systemic, but not mucosal hookworm-specific Th1 response (Gaze et al., 2012). Despite the predominance of Th2 cells and cytokines, and parasite-killing antibody isotypes (such as IgE), attempts to dislodge adult hookworms from the gut are mostly unsuccessful. Hookworms are potent inducers of regulatory immune responses that promote their survival and reproductive capacity. In humans, the expansion of regulatory T cells (Treg cells) has been reported (Wammes et al., 2014).

Regarding the use of probiotics to treat these infections, a study by Coêlho et al. analyzed the administration of *L. acidophilus* (ATCC 4536), *L. plantarum* (ATCC 8014), and *L. delbrueckii* (UFV H2B20) to control canine ancylostomiasis. The probiotic preparation was administered to naturally infected animals for 28 days, resulting in a reduction of eggs found in feces, as well as an increase in leukocyte and lymphocyte counts. It is important to mention that, before the treatment, red blood cells were below normal values in all dogs. The stabilization of the anemia in treated dogs, compared to its exacerbation in control animals, may be associated with the reduction of the number of eggs in the treated group (Coêlho et al., 2013).

H. polygyrus

H. polygyrus has a direct lifecycle with no intermediate hosts: eggs released in the feces of infected mice hatch in the environment producing L1 larvae, which molt twice to L3 larvae (infective stage), which are ingested by mice. L3 invade the intestinal mucosal layer molting into L4, which encyst in the muscle layer of the intestine and start maturing into adult parasites. Adult male and female worms mate in the lumen of the intestine, and gravid females produce eggs that are passed into feces.

H. polygyrus infection induces a strongly polarized Th2 response, which has been shown to be critical for the control and expulsion of the worm (Urban et al., 1991). A primary *H. polygyrus* infection induces IL-3, IL-4, IL-5, and IL-9 gene expression in the MLN and Peyer's patches (Svetić et al., 1993). Mononuclear cells from MLN, spleen or lamina propria stimulated *in vitro* with parasite antigens released high amounts of IL-4, IL-5, IL-9, IL-10, and IL-13 (Finney et al., 2007; Setiawan et al., 2007; Rausch et al., 2008). At the cellular level, infection is accompanied by expanded regulatory T cell populations and B cell hyperstimulation. In most mouse strains, these act to block protective Th2 immunity (Rick M. Maizels et al., 2012).

Reynolds et al. found that administration of *Lactobacillus taiwanensis* (BL263) enhanced Treg frequencies which made animals more susceptible to *H. polygyrus* infection. Moreover, *H. polygyrus* raises *Lactobacillus* species abundance in the duodenum of C57BL/6 mice, which are susceptible to *H. polygyrus* infection, but not in BALB/c mice, which are relatively resistant. Sequencing of samples at the bacterial gyrB locus identified the principal *Lactobacillus* species as *L. taiwanensis* (Reynolds et al., 2014). This causal relationship between commensal bacterium and *H. polygyrus*, highlights the importance of the mutualistic relationship between a commensal microbe and a helminth parasite, which provides a different perspective on the interactions in the intestinal tract that we have seen in this work.

Strongyloides spp.

Strongyloides spp. are soil-transmitted helminths. The primary mode of infection is through contact with soil contaminated with free-living larvae. The latter penetrate the skin and migrate through the body, eventually finding the small intestine where they mature into adults and produce eggs. Unlike other soil-transmitted helminths, the eggs of these helminths hatch into larvae in the intestine. Most of these larvae will be excreted in the stool, but some of them may mature and re-infect the host either by burrowing into the intestinal wall, or by penetrating the skin around the anus.

Like other helminth infections, strongyloidiasis elicits a predominant Th2 immune response (Wilkes et al., 2007). During primary infection neutrophils and eosinophils are attracted by parasite components and kill the larvae through the release of granule products. B-cells produce both IgM and IgG that collaborate with neutrophils to kill worms (Bonne-Année et al., 2011).

In the case of *Strongyloides* spp., a work by Oliveira-Sequeira et al. evaluated the administration of viable *B. animalis* strain 04450B before the infection with *S. venezuelensis*. They found in probiotic-treated mice a decrease in the worm burden (33%) and egg output (21%) accompanied by a reduced intestinal damage (Oliveira-Sequeira et al., 2014). Unfortunately, there is no immune analysis done in this study.

Haplorchis taichui

The intestinal trematode *Haplorchis taichui* is a medically important parasite infecting humans and livestock. This parasite has an aquatic life cycle, using freshwater snails as the first and cyprinid fish as the second intermediate hosts, with definitive hosts being fish-eating mammals (Dzikowski et al., 2004; Nithikathkul and Wongsawad 2008).

In a different approach to study the relationship between parasite infections and gastrointestinal microorganisms, Prommi et al. analyzed stool samples from 1,047 volunteers from Thailand. A parasitological study was conducted, and 16s rRNA sequencing was performed to evaluate microbial diversity. While this is not a study about the role of probiotics in parasite infections, it contributes to highlight the relevance of microbiota balance regarding parasite infection. A high prevalence of the trematode *Haplorchis taichui* was found in the samples. Notably, the group of volunteers without parasite infections exhibited a higher bacterial diversity (α diversity) compared with the *H. taichui*-infected group. Moreover, differences in bacterial community composition were also found (β diversity). The authors concluded that *H. taichui* infection modifies microbiome (Prommi et al., 2020). However, it is possible to question us: does parasite infection succeed if microbiota is first affected by other reasons? An alternative explanation, however, could be that *H. taichui* is more likely to produce successful infections in individuals with an intestinal microbiota previously affected for other reasons.

CONCLUSION

The use of probiotics as a treatment for helminth infection is an incipient research line with promising perspectives. Even though the papers discussed above show auspicious findings, they also raise additional questions. Regarding the host immune system, a detailed characterization of how the probiotic treatment affects the known immune mechanisms towards helminths is needed. As was mentioned before some features remain as open questions. For instance, which would be the best time to start treatment, right after the infection takes place or before? What is the optimal duration of the treatment? Once more, it would depend on the combination of parasite species and the probiotic strain (combination). Also, we have yet little information about which probiotics molecules, superficial or secreted, are responsible for the effects reviewed in this work. A fascinating aspect to explore would be the combined use of probiotics strains and anthelmintic drug treatments. Last but not least, it is important to highlight the lack of blind, placebo-controlled clinical trials as these treatments are expected to be applied to human or animal individuals.

We look forward to many more findings in this field as there is a wide range of possibilities to be explored that may deliver groundbreaking treatment strategies for helminth diseases.

AUTHOR CONTRIBUTIONS

MS conceptualized the content of the manuscript. MS initiated the draft, and CV and DM contributed several sections. MS, CV, PB, and DM edited and revised the manuscript.

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REVIEW
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Gut Microbiota Modulation as a Potential Target for the Treatment of Lung Infections

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The gastrointestinal and respiratory systems are colonized by a complex ecosystem of microorganisms called the microbiota. These microorganisms co-evolved over millions of years with the host, creating a symbiotic relationship that is fundamental for promoting host homeostasis by producing bioactive metabolites and antimicrobial molecules, and regulating the immune and inflammatory responses. Imbalance in the abundance, diversity, and function of the gut microbiota (known as dysbiosis) have been shown to increase host susceptibility to infections in the lungs, suggesting crosstalk between these organs. This crosstalk is now referred to as the gut-lung axis. Hence, the use of probiotics, prebiotics, and synbiotics for modulation of gut microbiota has been studied based on their effectiveness in reducing the duration and severity of respiratory tract infections, mainly owing to their effects on preventing pathogen colonization and modulating the immune system. This review discusses the role and responses of probiotics, prebiotics, and synbiotics in the gut-lung axis in the face of lung infections.

Keywords: symbiotics, mucosal immmunity, gut-lung axis, prebiotcs, probiotics, immunobiotics, inflammation, microbiota

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INTRODUCTION

Microorganisms and humans have co-evolved for thousands of years, and many survival functions have been defined throughout this time for both. All body surfaces are colonized by complex and dynamic communities of symbiotic microorganisms, including bacteria, viruses, fungi, helminths, and protists, called microbiota (Grice and Segre, 2012; Sender et al., 2016). As demonstrated by next-generation sequencing, the lungs and gut possess unique microbiota that differ mainly in composition and structure, with bacteria being the most predominant microorganisms (Dickson et al., 2015; Santacroce et al., 2020). The microbiota plays fundamental roles in host homeostasis via the metabolism of nutrients, production of vitamins, metabolites, and antimicrobial molecules, activation of the immune system, and regulation of the inflammatory process (Dang and Marsland, 2019). The gut dysbiosis has been shown to increase susceptibility to infection in the lungs, and infections in the lung are identified as a cause of gut dysbiosis; highlighting a bidirectional link between these two organs; this crosstalk is now called the gut-lung axis (Li et al., 2008; Hand et al., 2016; Budden et al., 2017; Sencio et al., 2020). Also, the lung and gut originate from the same embryonic organ, the foregut, and consequently have some structural similarities that might

Abbreviations: GF, Germ-free; GPCR, G-protein-coupled receptors; ICU, Intensive care units; IFV, Influenza virus; PRR, attern recognition receptors; PVM, Pneumonia virus of mice; ROS, Reactive oxygen species; RSV, Respiratory syncytial virus; SCFA, Short-chain fatty acids; SFB, Segmented filamentous bacteria; URT, Upper respiratory tract; VAP, Ventilator-associated pneumonia.

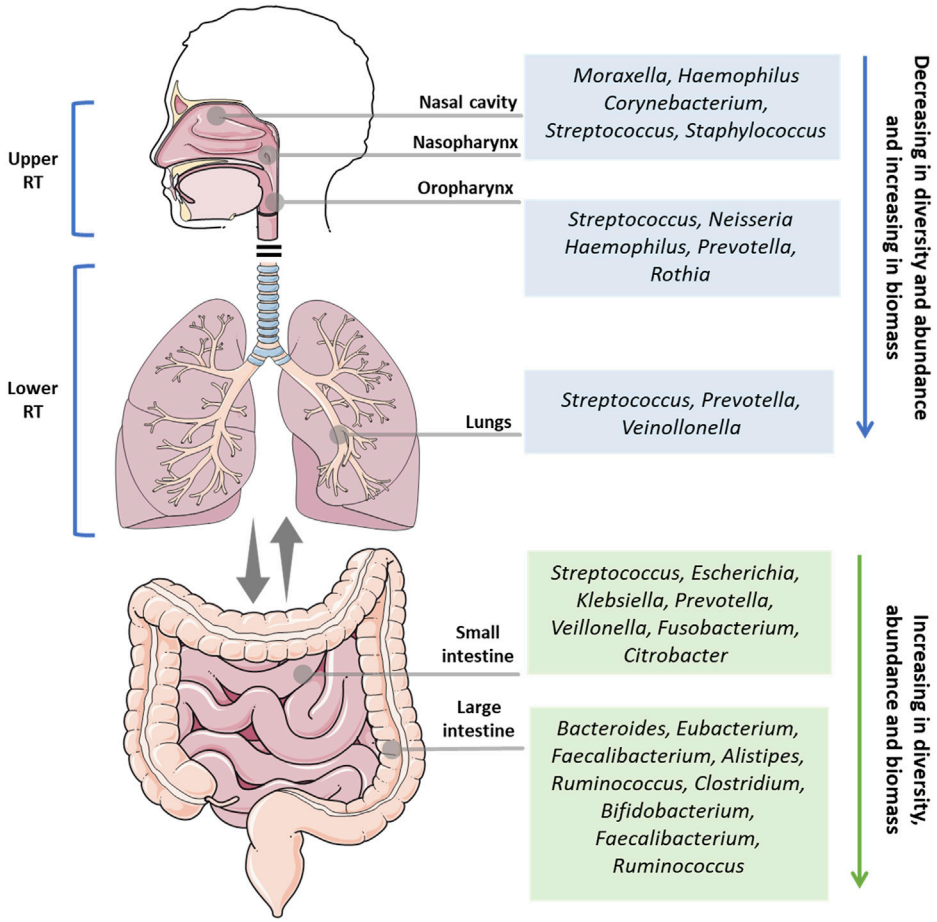

FIGURE 1 | Overview of the main microbial genus in the health upper respiratory tract (nasal cavity, nasopharynx, oropharynx), lower respiratory tract (lungs), small intestine, and large intestine. RT: respiratory tract.

contribute to the interaction between these two organs (Faure and de Santa Barbara, 2011). Respiratory tract infections (RTI) are a global health concern. Approximately 2.38 million deaths were attributed to RTI in 2016 alone, making it the sixth leading cause of mortality among all ages and the leading cause of death among children under 5 years (Ferkol and Schraufnagel, 2014; Troeger et al., 2018). Given the importance of the gut-lung axis, our review summarized the latest experimental and clinical studies on this topic and showed that modulation of the gut-lung axis with probiotics, prebiotics, and synbiotics, could be an important therapeutic target for preventing and treating lung infections caused by bacteria, viruses, fungi, and parasites.

GUT-LUNG AXIS IN RESPIRATORY TRACT INFECTIONS

The respiratory system is composed of different organs, and is divided into two main parts: the upper respiratory tract (URT) and the lower respiratory tract (LRT). The URT comprises the nostrils, nasal passages, paranasal sinuses, nasopharynx, and oropharynx, while the lower respiratory tract comprises the trachea, bronchi, bronchioles, and alveoli. These organs make up one of the largest surface areas in the human body, that from the nostrils to the lungs, is colonized by a symbiotic and diverse community of microorganisms (**Figure 1**).

The microbiota of the lungs and gut of healthy individuals differ significantly in taxonomic composition, diversity, and function. In contrast to the thriving resident microbiota in the gut, the lung microbiota is composed of transient microorganisms mainly derived from URT. While Bacteroidetes and Firmicutes are the most abundant bacterial phyla in both microbiotas, the lung and gut microbiota are very different at the species level. In the lungs, the genera *Streptococcus* spp., *Veillonella* spp., and *Prevotella* were the most abundant, whereas *Bacteroides*, *Faecalibacterium*, and *Bifidobacterium* are more prevalent in the gut (Sender et al., 2016). In a disease or dysbiotic state, other organisms are present in the lung, such as viruses, including human rhinovirus, human bocavirus, polyomaviruses, human adenovirus, and human coronavirus, and fungi such as *Aspergillus* spp., *Penicillium* spp., *Candida* spp., and *Alternaria* spp. (Papadopoulos and Skevaki, 2006; Limon et al., 2017).

The immune responses in the gut-lung axis depend on the balance of microbiota composition, particularly in the gut. The regulated interaction between the metabolites and antigens of symbiotic microbiota with the host is crucial for the activation of pattern recognition receptors (PRRs) and metabolic sensor receptors such as G-protein-coupled receptors (GPCRs), and the production of inflammatory mediators, which are necessary for the migration, activation, and proliferation of innate and adaptive immune cells responsible for the production of pro-and anti-inflammatory cytokines, immunoglobulins, and antimicrobial peptides (Fan and Pedersen, 2021). These cells and molecules can move bidirectionally between the lungs and the gut through the bloodstream and lymphatic system and regulate immune and inflammatory responses (Marsland et al., 2015; Dang and Marsland, 2019).

Intestinal dysbiosis is responsible for increasing the susceptibility of the host to lung disease, as evidenced by the high prevalence of asthma in patients with irritable bowel syndrome (Yazar et al., 2001). Experimentally, mice treated with antibiotics are more susceptible to lethal infection by the influenza virus (IFV) (Ichinohe et al., 2011; Pang et al., 2018). Furthermore, infections in the lungs are also linked to dysbiosis in the gut; mice infected with IFV displayed a significant increase in *Enterobacteriaceae* and decreased diversity of *Lactobacillus* and *Lactococcus* (Wang et al., 2018). Influenza infection also affects the production of short-chain fatty acids (SCFAs) and impairs the gut barrier properties thereby increasing susceptibility to second bacterial infections (Sencio et al., 2020, 2021).

SCFAs, such as butyrate, propionate, and acetate derived from the fermentation of dietary fibers by the microbiota, are involved in regulating the inflammatory process and pulmonary immune response (Fukuda et al., 2011; Trompette et al., 2014). SCFAs activate GPCRs and inhibit histone deacetylases, thus contributing to the reduction of inflammation in the gut-lung axis by inhibiting the NF-κB signaling pathway, increasing regulatory T (Treg) cells, and decreasing T helper 1 (Th1) and Th17 cells (Maslowski et al., 2009; Kim et al., 2013; Li et al., 2018). SCFAs can also reach the bone marrow and influence the generation and development of immune cells such as Ly6C- and Ly6C + monocytes and dendritic cells, which can be recruited into the lungs and modulate the immune response against pathogens (Trompette et al., 2014, 2018; Kopf et al., 2015). Our research group has also demonstrated that activation of the GPR43 receptor in neutrophils and alveolar macrophages by acetate is essential for modulating the inflammatory response and controlling pulmonary infection by *Klebsiella pneumoniae* (Galvão et al., 2018) and *Streptococcus pneumoniae* serotype 1 in mice (Sencio et al., 2020). In another study, activation of GPR43 in pulmonary epithelial cells induced interferon (IFN)-β in the lungs and increased the protection of mice infected with respiratory syncytial virus (RSV) (Antunes et al., 2019).

PROBIOTICS, PREBIOTICS, AND SYNBIOTICS

Probiotics are live microorganisms that confer benefits to the host when administered in adequate amounts (Salminen et al., 2021). Probiotics are considered important tools for the modulation of microbiota in the gut-lung axis, with their benefits on the gut-lung axis dependent on the strains used (**Figure 2**). However, common mechanisms have been reported between species, such as –1) colonization of the respiratory and intestinal tracts, 2) production of SCFAs and antimicrobial peptides, 3) maintenance of the integrity of the intestinal and pulmonary mucosa, and –4) stimulation of the innate and adaptive immune system (Bermudez-Brito et al., 2012; Salminen et al., 2021). The benefits of probiotics have been shown in animal models and clinical studies in many disease conditions, such as post-antibiotic-associated diarrhea, allergies and inflammatory bowel diseases, and respiratory tract infections (Vieira et al., 2013). For a given microorganism to be assessed as a probiotic, biosafety criteria and scientific evidence regarding its biological benefits must be considered (Harzallah and Belhadj, 2013). *Lactobacillus* and *Bifidobacterium* species are more commonly used as probiotics; however, yeasts, certain *Streptococcus* spp. strains, and *Bacillus* spp. are also used as probiotics, but less frequently (Fijan, 2014). The use of inactivated probiotics is also of great interest because live probiotic microorganisms may cause systemic infections, excessive immune stimulation, and antibiotic resistance gene transfer (Doron and Snydman, 2015). Taking this into consideration, the term postbiotics was proposed as preparation for inanimate microorganisms and/or their components that confer a health benefit on the host (Salminen et al., 2021).

Prebiotics are dietary fibers, such as inulin, fructooligosaccharides, and galactooligosaccharides, which are fermented in the gut and promote an increase in the diversity and activity of specific symbiotic microorganisms (Salminen et al., 2021). The activity of prebiotics also leads to an enhancement of immune response, decrease in colon pH, local induction of reactive oxygen species (ROS), trophic effects on enterocytes, and anti-inflammatory responses (Vieira et al., 2013). In addition, the SCFAs butyrate and propionate, derived from the metabolism of prebiotics, can increase miRNAs through the inhibition of histone deacetylases, leading to improved antibody class switching and local and systematic impact on the T-dependent and T-independent immunoglobulin production (Sanchez et al., 2020).

Synbiotics consist of probiotics and prebiotics to achieve synergistic and complementary effects on their functions (Salminen et al., 2021). A recent meta-analysis of randomized controlled clinical trials involving over 10,000 individuals showed the effectiveness of synbiotic interventions in reducing the rate of respiratory tract infections (Chan et al., 2021). Understanding the specific mechanisms of interaction between probiotics and prebiotics and their modulation of the gut environment and immune response will lead to better utilization of the synbiotics to treat infections and metabolic diseases.

Gut-Lung Axis Modulation in the Context of Bacterial Lung Infections

The lung is highly vulnerable to bacterial infections due its constant exposure to environment agents. One of the most

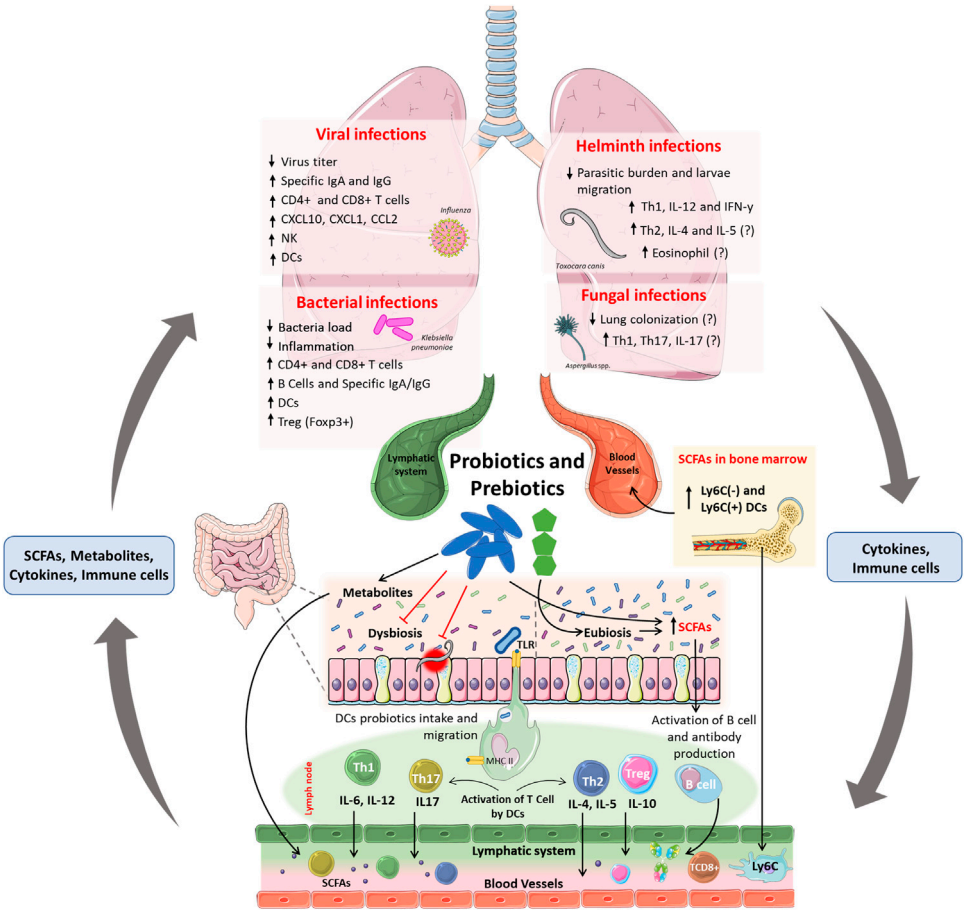

FIGURE 2 | Effects and mainly mechanisms of probiotics and prebiotics in the gut-lung axis and context of respiratory infections. Probiotics and prebiotics administered orally can improve dysbiosis and induce eubiosis in the host, leading to an increase in SCFAs directly (produced by probiotics) or indirectly (produced by commensal microbiota). Furthermore, probiotics can also reduce the burden and epithelial damage induced by intestinal parasites. The uptake of probiotics by DCs in the intestinal submucosa, and their migration to lymph nodes, induces the activation and proliferation of Th1, Th2, Th17, Treg, and B cells. Activated T cells and B cells produce cytokines and antibodies, enter the circulatory and lymphatic systems, and reach the lungs, where they will increase resistance to infections caused by viruses, bacteria, and fungi. The fermentation of prebiotics and production of SCFAs increases the number of DCs precursors in the bone marrow and increases $CD8^+$ T cells activity, that confer protection against infections in the lung. The immunomodulation demonstrated after the administration of probiotics and prebiotics may be linked to the reduced viral titer, bacterial colonization, parasite load, and migration in the lungs. Probiotic-induced immunomodulation can increase the frequency of dendritic cells and $CD4^+$ and $CD8^+$ T cells in the lungs against infections by viruses and bacteria and can increase specific IgG and IgM antibodies to these pathogens. Also, the increase in Treg cells may be related to the reduction of inflammation-induced lung damage. In parasitic infections, probiotics have been linked with increased frequency of Th1 and concentration of IL-12 and IFN-γ, which may justify the reduction in the parasite load and larvae migration in the lung. Because there are no scientific studies that demonstrate the reduction of lung colonization by fungi after oral administration of probiotics, is still unknow if the antimycotic potential from probiotics metabolites, as shown *in vitro*, could be applied in an *in vivo* system.

common diseases caused by bacteria in the lungs is pneumonia that is characterized by alveolar infection and intense inflammatory response that ranges from mild to severe and can affect both the right and left lobes and may impair the gaseous exchange. The most common causes of bacterial pneumonia in immunocompetent hosts include *S. pneumoniae*, *Haemophilus* spp., and *Mycobacterium tuberculosis*. In immunocompromised hosts the number of pathogens that cause pneumonia is much larger, and in general those individuals are more vulnerable and have worse outcomes (van der Poll and Opal, 2009) (**Table 1**).

Several studies have shown that the oral administration of different strains of probiotics, such as *Lactobacillus bulgaricus* CRL 423 and *Streptococcus thermophilus* CRL 412 (Villena et al., 2006), *L. casei* CRL 431 (Villena et al., 2005, 2009), *L. fermentum* (Cangemi De Gutierrez et al., 2001), and *L. rhamnosus* CRL 1505 (Barbieri et al., 2017) causes: 1) increased resistance to infection, 2) decreased number of bacteria in the lungs, and 3) increased survival of mice infected with *S. pneumoniae*. In general, these articles, associated this protection with an increase in neutrophils, lymphocytes, macrophages, phagocytic activity, and levels specific anti-*S. pneumoniae* IgG and IgA in the lungs. The increase in phagocytic activity and the number of neutrophils in the lower respiratory tract is the first line of defense against invading pathogens, and the increase in regulatory cells and cytokines contributes to the reduction of the inflammatory

TABLE 1 | Pre-clinical studies on the modulation of the microbiota for treatment of bacterial and viral lung infections.

Strategy for microbiota modulation	Dose and route of administration	Experimental model	Pathogen	Main outcomes	References
Effects on bacterial pathogen					
Lactobacillus bulgaricus CRL 423 and *Streptococcus thermophilus* CRL 412	2×10^8 CFU, via oral	Malnourished, Swiss albino mice	*Streptococcus pneumoniae*	Reduced bacterial load in the lungs; increased bactericidal function of bronco-alveolar phagocytes; reduced tissue inflammation; increased neutrophils in blood; and increased level of lung anti-pneumococcal IgA and IgG	Villena et al. (2006)
Lactobacillus casei CRL 431	1×10^9 CFU, via intranasal	Malnourished, Male, 3-week-old Swiss albino mice	*Streptococcus pneumoniae*	Increased the bacteria lung clearance; improved production of TNF-α; increased activity of phagocytes in the respiratory tract; increased IL-4, IL-10, and *Pneumococcus*-specific IgG	Villena et al. (2009)
Lactobacillus casei CRL 431	1×10^9 CFU, via oral	Male 6-week-old Swiss albino	*Streptococcus pneumoniae*	Increased pathogen clearance from blood; lower lung damage; improved number of leukocytes and neutrophils; and increased levels of antipneumococcic IgA in BAL.	Villena et al. (2005)
Lactobacillus fermentum	1×10^7, via intranasal	Adult, BALB/c mice	*Streptococcus pneumoniae*	Increased the number of activated macrophages and lymphocytes; and increased anti-*S.pneumoniae* antibodies	Cangemi De Gutierrez et al. (2001)
Lactobacillus rhamnosus CRL1505	1×10^8 CFU, via intranasal	Malnourished, Male, 3-week-old Swiss-albino mice	*Streptococcus pneumoniae*	Changed the quantitative and qualitative alterations of $CD4^+$ T cells in the bone marrow, thymus, spleen and lung induced by malnutrition and infection; and increased IL-10 and IL-4 in respiratory and systemic compartments	Barbieri et al. (2017)
Lactobacillus casei CRL 431, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*	1×10^9 CFU, via oral	3-week-old, Swiss albino mice	*Pseudomonas aeruginosa*	Enhanced lung clearance of *P. aeruginosa*; increased phagocytic activity of alveolar macrophages; and increased IgA and IgM levels in BAL.	Alvarez et al. (2001)
Lactobacillus rhamnosus GG	4×10^8 CFU, via oral	5 to 8-week-old, FVB/N mice	*Pseudomonas aeruginosa*	Mice treated had improved survival; reduced bacterial counts in BAL; decreased the levels of IL-6 and increased levels of IL-10 mRNA; improved lung pathology; and increased levels of Treg cell marker Foxp3	Meitert et al. (2013)
Lactobacillus fermentum K.C6.3.1E, *Lactobacillus zeae* Od.76, and *Lactobacillus paracasei* ES.D.88	9×10^6 CFU, via intratracheal	6 to 8-week-old, C57BL/6 mice	*Pseudomonas aeruginosa*	Decreased secretion in BAL of IL-6 and TNF-α	Fangous et al. (2019)
Bifidobacterium longum 5^{1A}	1×10^8, via oral	8 to 12-week-old, C57BL/6 WT and Mal/$TIRAP^{-/-}$ mice	*Klebsiella pneumoniae*	Reduced bacterial burden; faster resolution of inflammation; decreased lung damage; increased production of IL-10; and increased alveolar macrophages ROS associated with Mal/TIRAP activation	Vieira et al. (2016)
Effects on viral pathogen					
Lactobacillus casei Shirota	1×10^8 CFU, via oral	Neonatal and infant, BALB/c mice	Influenza A/PR/8/34 (H1N1)	Higher survival rate, reduced titer of virus in the nasal washings; greater pulmonary NK cell activity; and increased IL-12 production by mediastinal lymph nodes	Yasui et al. (2004)
Lactobacillus rhamnosus M21	1×10^9 CFU, via oral	Female, specific pathogen-free, BALB/c mice	Influenza A/NWS/33 (H1N1)	Increased IL-2 and IFN-γ; increased sIgA levels; reduced inflammatory cells in BAL.	Song et al. (2016)

(Continued on following page)

TABLE 1 | (*Continued*) Pre-clinical studies on the modulation of the microbiota for treatment of bacterial and viral lung infections.

Strategy for microbiota modulation	Dose and route of administration	Experimental model	Pathogen	Main outcomes	References
Lactobacillus pentosus S-PT84	Heat-killed *L. pentosus* S-PT84, via intranasal	Female, BALB/c mice	Influenza A/PR/8/34 (H1N1)	Increased survival rates; reduced titer of influenza virus in BAL; increased IL-12 and IFNγ production in mediastinal lymph node cells; increased IL-12 and IFN-α in BAL; and increased NK cell activity	Izumo et al. (2010)
Lactobacillus plantarum and *Leuconostoc mesenteroides*	1×10^9 CFU, via oral	Female, 5-week-old, BALB/c mice	Influenza rK09 (H1N1)	Restrained viral replication; and increased rates of survival of infected mice	Bae et al. (2018)
Lactobacillus rhamnosus GG	200 μg of *L. rhamnosus* GG lyophilized, via intranasal	Female, 7-week-old BALB/c mice	Influenza A/PR/8/34 (H1N1)	Higher survival rate; increased cell-killing activity of lung cells; and increased mRNA expression of interleukin IL-1 beta, TNF and MCP-1	Harata et al. (2010)
Lactobacillus acidophilus L-92	4×10^{10} CFU, via oral	Female, 4-weeks-old BALB/c mice	Influenza A/PR/8/34 (H1N1)	Reduced Virus titers in the lung; increased NK cells activity; decreased the number of neutrophils; increased eotaxin, MCSF, IL-1β, RANTES and IFN-α in the lung; and increased IL-17 levels in Peyer's patches	Goto et al. (2013)
Lactobacillus plantarum DK119	1×10^8 or 1×10^9 CFU, via oral	Female, BALB/c mice	Influenza A/PR8	Reduced lung viral loads; increased levels of cytokines IL-12 and IFN-γ in BAL; and reduced degree of inflammation	Park et al. (2013)
Lactococcus lactis subsp. *lactis* JCM5805	1 mg of heat-killed *L. lactis* subsp. *lactis* JCM5805, via oral	Female, DBA/2jjcl mice	Murine Parainfluenza virus (mPIV1)	Increased survival rate; prevention of weight loss; reduced lung histopathology scores; increased activation of Peyer's patches (PP) and PP pDCs; increased levels of type I IFNs; and increased expressions of anti-viral factors such as *Isg15*, *Oasl2*, and *Viperin*, at lung	Jounai et al. (2015)
Lactobacillus paracasei CNCM I-1518	2×10^8 CFU, via oral	Female, 6-week-old BALB/c mice	Influenza A/Scotland/20/74 (H3N2)	Reduced weight loss; and increased recruitment of inflammatory myeloid cells, such as interstitial monocytes and dendritic cells, to the lungs	Belkacem et al. (2017)
Bacillus subtilis 3 (UCM B-5007)	1×10^7 CFU, via oral	Four-week-old BALB/c mice	Influenza A/FM/1/47 (H1N1)	Prevented influenza infection	Starosila et al. (2017)
Lactobacillus rhamnosus CRL1505	1×10^8 CFU, via oral	Male, 6-week-old BALB/c mice	Respiratory Syncytial Virus strain A2 and Influenza virus A/PR/8/34 (H1N1)	Reduced lung immune-coagulative reaction triggered by TLR3 activation	Zelaya et al. (2014)
Lactobacillus plantarum NCIMB 8826 and *Lactobacillus reuteri* F275	1×10^9 CFU, via intranasal	BALB/c and C57BL/6 MyD88$^{-/-}$ mice	Pneumonia Virus of mice (PVM) strain J3666	Protection against lethal infection; reduced granulocyte recruitment; reduced expression of proinflammatory cytokines CXCL10, CXCL1, CCL2, and TNF.	Gabryszewski et al. (2011)
Lactobacillus rhamnosus CRL1505 and CRL1506	1×10^8 CFU, via intranasal	Female, 3-week-old BALB/c mice	Human RSV strain A	Increased levels of IFN-α, IFN-β, IFN-γ, IL-6 and IL-10; increased levels of $CD4^+$ Tregg cells and $CD11c^+CD103^+$ DCs; reduced viral replication and lung damage	Tomosada et al. (2013)

response and to the maintenance of tolerance to symbiotic microorganisms, which is necessary to reduce damage associated with infections by pathogens (Martin and Frevert, 2005).

Similar results were observed in mice infected with *Pseudomonas aeruginosa*, treated orally with the probiotics *L. casei* CRL 431, *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus* (Alvarez et al., 2001) and *L. rhamnosus* GG (Meitert et al., 2013).

TABLE 2 | Clinical studies on the modulation of the microbiota for treatment of bacterial and viral lung infections.

Strategy for microbiota modulation	Dose and route of administration	Study design and subjective	Pathogen and disease	Outcome	References
Effects on bacterial pathogen					
Lactobacillus casei rhamnosus Lcr35	1×10^9 CFU, via oral	Prospective, randomized, double-blind, placebo-controlled pilot study with patients aged 18-91	*Pseudomonas aeruginosa*	Reduction in the occurrence of *P. aeruginosa* respiratory colonization and/or infection in the probiotic group. Reduction in the frequency of VAP to *P. aeruginosa*	Forestier et al. (2008)
Lactobacillus rhamnosus GG	2×10^9 CFU, via oral	Prospective, randomized, double-blind, placebo-controlled trial with patients at high risk of developing VAP	VAP by Gram-positive and Gram-negative pathogens	Reduction in the development of microbiologically confirmed VAP. Patients treated with probiotics had fewer days of antibiotics prescribed for VAP.	Morrow et al. (2010)
Bifidobacterium breve Yakult, *Lactobacillus casei* Shirota, and galacto-oligosaccharide	1×10^8 CFU of *B. breve* and *L. casei* and Galacto-oligosaccharides in a 10 g/day formula, via oral	Randomized controlled trial with patients with more than 16 years old	Patients with more than 16 years old, placed on a ventilator, and who were diagnosed as having sepsis	Reduced incidence of VAP. Increased number of *Bifidobacterium* and *Lactobacillus*. Increased concentration of acetate in the feces	Shimizu et al. (2018)
Effects on viral pathogens					
Lactobacillus rhamnosus GG ATCC 5310 and galacto-oligosaccharide and polydextrose mixture	1×10^9 CFU/day for 1–30 days and 2×10^9 CFU/day for 31–60 days, via oral	Randomized, double-blind, placebo-controlled study with preterm infants	Rhinovirus-associated respiratory tract infection	Reduced respiratory tract infections. Reduced rhinovirus-induced episodes	Luoto et al. (2014)
Lactobacillus brevis KB290	6×10^9, via oral	Open-label, parallel-group trial with elementary schoolchildren	Influenza infection	Reduced incidence of influenza infections	Waki et al. (2014)
Lactobacillus rhamnosus GG	1×10^9, via oral	Randomized, double-blind, placebo-controlled study with nursing home residents aged 65 and older	Influenza infection	Reduced laboratory-confirmed respiratory viral infections	Wang et al. (2018)
Streptococcus thermophilus DSM 32245, Bifidobacterium lactis DSM 32246, Bifidobacterium lactis DSM 32247, Lactobacillus acidophilus DSM 32241, Lactobacillus helveticus DSM 32242, Lactobacillus paracasei DSM 32243, Lactobacillus plantarum DSM 32244, and Lactobacillus brevis DSM 27961	2.4×10^9, via oral	Retrospective, observational cohort study with adults	COVID-19 pneumonia by SARS-CoV-2	Increased survival rates of patients that received BAT plus oral bacteriotherapy	Ceccarelli et al. (2021)

In addition, the administration of *L. rhamnosus* GG induces an anti-inflammatory response by increasing the levels of regulatory T cells (Treg) Foxp3+ and decreasing the production of the proinflammatory cytokine IL-6. This anti-inflammatory profile was also observed in mice infected with *P. aeruginosa* with intratracheal administration of probiotics *L. fermentum* K.C6.3.1E, *L. zeae* Od.76, and *L. paracasei* ES.D.88, demonstrated by the reduction of lung inflammation and decreased production of IL-6 and tumor necrosis factor (TNF)-α (Fangous et al., 2019).

In an experimental lung infection by *K. pneumoniae*, the administration of viable or inactivated probiotic *Bifidobacterium longum* 5^{1A} induced pulmonary clearance of *K. pneumoniae* by increasing ROS production in alveolar macrophages through activation of the mal/TIRAP signaling pathway (Vieira et al., 2016). There was a concomitant reduction in the inflammatory process and concentration of cytokines TNF-α and IL-6 and an increase in IL-10 in the lungs of mice. However, only viable probiotics were able to increase the levels of IL-10 in the lungs of mice. Viable *B. longum* 5^{1A} produces SCFA acetate in large quantities, and acetate administration in mice before respiratory infection by *K. pneumoniae* induced increased production of IL-10 in animals. The authors demonstrated that acetate might be the primary inducer production of IL-10 in this model (Vieira et al., 2016). In addition, intestinal colonization of germ-free mice with *B. longum* 5^{1A} restored the ability of these mice to decrease infection by increasing the production of CXCL1 and the recruitment of

neutrophils (Vieira et al., 2016). In mice infected with IFV, the lower quantity of acetate was also related to increased susceptibility to secondary respiratory pneumococcal infection, mainly due to the impaired bactericidal activity of alveolar macrophages, and this detrimental effect was restored after acetate supplementation (Sencio et al., 2020).

The potential of probiotics to protect the host from pulmonary infections has also been assessed in several clinical studies, including diverse patients, methodological designs, and inclusion criteria (**Table 2**). Most of these studies focused on probiotics for prevention and treatment of nosocomial pulmonary infections in patients admitted to intensive care units (ICUs). Two prospective, randomized, double-blind, and placebo-controlled studies showed that the probiotics *L. casei rhamnosus* Lcr35 and *L. rhamnosus* GG, administered orally or oropharyngeally, resulted in decreased colonization and infection of the LRT by *P. aeruginosa* or related gram-positive and gram-negative pathogens in patients admitted to the ICU using mechanized pulmonary ventilation (Forestier et al., 2008; Morrow et al., 2010). Only one study showed that administration of a synbiotic consisting of *B. breve* Yakult, *L. casei* Shirota, and galactooligosaccharides decreased the incidence of ventilator-associated pneumonia (VAP) in patients diagnosed with sepsis admitted to the ICU (Shimizu et al., 2018).

In general, we can conclude that the modulation of the intestinal microbiota, mainly with probiotics, is an exciting alternative for treating lung diseases caused by bacteria. Although it is already well established in the literature that probiotic species and strains behave differently according to their metabolic pathways and their interaction with the host, the probiotic species used against lung alterations attract attention to those from the *Lactobacillus* genus. Most articles demonstrated that non-specific immune responses mediated by probiotics, prebiotics, and symbionts are the principal host protection against lung bacteria. Generally, it seemed more significant phagocytic activity of lung macrophages, reduced lung bacterial load, and less tissue inflammation, associated with increased levels of IL-4 and IL-10, increased frequency of Treg cells, increased production of IgA and IgG, and reduced of IL-6 and TNF-α levels. This demonstrates a more resolving anti-inflammatory profile after modulation of the host's intestinal microbiota. Despite the benefits, these articles use different study designs, experimental models, doses, and routes of administration, making it challenging to translate the results obtained in animal models to humans and thus develop more specific therapies with probiotics.

Gut-Lung Axis Modulation in the Context of Lung Viral Infections

Viral infections generally cause common cold, bronchiolitis, and pneumonia and vary widely in severity depending on age, immune and nutritional status, genetics, and use of antibiotics. IFV, RSV, and rhinovirus are the most abundant and common causes of lung infections (Jain, 2017). IFV is well known to cause outbreaks of varying severity every year, but recently the novel coronavirus SARS-CoV-2 has emerged as a pandemic that has caused more than 3.5 million deaths (Zhu et al., 2020) (**Table 1**).

Several studies have demonstrated the potential for oral and intranasal administration of probiotics such as *L. casei* Shirota (Yasui et al., 2004), *L. rhamnosus* M21 (Song et al., 2016), *L. pentosus* S-PT84 (Izumo et al., 2010), and *L. plantarum* and *Leuconostoc mesenteroides* (Bae et al., 2018) to protect and increase the survival of IFV-infected animals, mainly by inducing anti-viral immune responses with the activation of NK cells and increased production of cytokines such as IL-12 and IFN-γ, increased production of IgA in the respiratory mucosa, and reduction of polymorphonuclear inflammatory infiltrate in the lung tissue. In addition to these protective effects, *L. rhamnosus* GG administered intranasally (Harata et al., 2010), and *L. acidophilus* L-92 (Goto et al., 2013) also demonstrated the ability to increase the levels of proinflammatory cytokines, such as IL-1β, monocyte chemotactic protein 1 (MCP-1), and chemokines such as eotaxin and M-CSF.

Dendritic cells are crucial for developing immune responses because of their ability to detect pathogens through TLRs and create a link between innate and adaptive immune responses. Mice with depleted alveolar macrophages lost the anti-viral protection against IFV infection conferred by increasing IL-12 and IFN-γ levels after oral administration of the probiotic *L. plantarum* DK119 (Park et al., 2013). In addition, the importance of dendritic cells was demonstrated after oral administration of the probiotic *Lactococcus lactis* subsp. *lactis* JCM5805 in mice infected with murine parainfluenza virus. The authors showed that the probiotic was incorporated into $CD11c^+$ immune cells in Peyer's patches and activated plasmacytoid dendritic cells that produce type I IFNs at draining mucosal sites. The authors also observed an increase in IFN-related genes, such as *lsg15*, *Oasl2*, and *Viperin* in the lungs, suggesting that the type I IFN produced by plasmacytoid dendritic cells could reach systemic levels and induce anti-viral activity in the lungs. In addition, *ex vivo* stimulation with murine parainfluenza virus of lung lymphocytes from mice treated with JCM5805 demonstrated high expression of IFN-α and IFN-β (Jounai et al., 2015).

Determining the taxonomic composition and function of the microbiota is crucial for understanding the impact of probiotics on the protective response against pathogens. The oral administration of *L. paracasei* CNCM I-1518 did not modify the gut microbiota structure in mice infected with IFV; however, it conferred protection against the virus (Belkacem et al., 2017).

Diets rich in inulin and SCFAs improve mice lung pathology after infection with IFV by promoting the differentiation of alternatively activated macrophages (AAMs) from circulating Ly6C- monocytes and decreasing the immunopathological effects of neutrophils. Also SCFAs increases anti-IFV immunity by enhancing the $CD8^+$ T cells activity by serving as a substrate for fatty acid oxidation and by specifically interacting with the receptor GPR41 (Trompette et al., 2018). One study showed that activation of GPR43 and interferon-α/β receptor (IFNAR) in pulmonary epithelial cells by SCFA acetate induced increased levels of IFN-β in the lungs and increased protection of mice in an experimental model of RSV infection (Antunes et al., 2019).

Other probiotic-derived metabolites also require further investigations. A peptide P18 produced by the probiotic *Bacillus subtilis* 3 (UCM B-5007) share high structural homology with IFV neutralizing antibody, and it is capable of inhibit IFV replication *in vitro* and protect 80% of mice from lethal IFV infection when administered in a therapeutic regimen. This protection is superior to that observed using the anti-viral drug oseltamivir (approximately 70%) (Starosila et al., 2017).

Double-stranded RNA intermediates from IFV and RSV are associated with changes in the host's coagulation process by activation of receptors such as TLR-3, and retinoic acid-inducible gene I (RIG-I). The activation of these receptors by these viruses, increases the expression of coagulation factors in endothelial cells and monocytes and inhibits fibrinolysis, inducing a prothrombotic state in the hosts, leading to fibrin deposition in the pulmonary alveoli and exacerbation of tissue inflammation. In order to address this issue one study demonstrated in a murine model of IFV and RSV infection, that oral administration of *L. rhamnosus* CRL 1505 in mice increases the clearance of both viruses and controls immune-coagulative responses initiated by the activation of TLR-3 in the lungs, in a process dependent on IL-10 (Zelaya et al., 2014).

Intranasal administration of viable or heat-killed *L. plantarum* NCIMB 8826 and *L. reuteri* F275 protected mice from lethal pneumonia virus of mice (PVM) infection. The lungs showed minimal inflammation, with fewer granulocytes and an increased number of lymphocytes, correlated with a reduction in proinflammatory cytokines CXCL10, CXCL1, CCL2, and TNF-α. Evaluation of the lymphocyte populations demonstrated that treatment did not result in changes in the relative proportions of $CD4^+$ T cells ($CD3^+CD4^+CD8^-$), $CD8^+$ ($CD3^+CD4^-CD8^+$), or B cells ($B220^+$). In contrast, the fraction of NK cells ($CD3^-DX5^+$) decreased. The authors demonstrated that these results are not specific for *L. plantarum* NCIMB 8826 and *L. reuteri* F275, as the same protection was observed when using the non-pathogenic gram-positive bacteria *Listeria innocua*. These probiotics also increased the survival of mice infected with the PVM, with the deleted MyD88 (TLRs adapter protein) gene ($MyD88^{-/-}$), thus demonstrating that this induced protection can be TLR-independent (Gabryszewski et al., 2011). However, other studies have determined that the anti-viral activity of probiotics is related to the activation of TLRs. This was demonstrated in mice with *L. rhamnosus* probiotics CRL1505 and CRL1506 that differentially activate the TLR3/RIF-I pathway to inoculate with poly (I:C) (a molecular pattern associated with viruses). The activation of TLR3/RIF-I leads to increased production of IFN-γ, IFN-β, TNF-α, IL-6, and IL-10, the frequency of $CD3^+CD4^+IFN\text{-}\gamma^+$, $CD3^+CD4^+IL\text{-}10^+$, and the dendritic cells $D11c^+CD11b^{low}CD103^+$ and $CD11c^+CD11b^{high}CD103$, in the lungs. Additionally, this study showed an increase in MHC-II levels in both populations of dendritic cells. The authors also demonstrated that this protective response and modulation of the immune response were similar to those observed in mice infected with the human RSV strain (Tomosada et al., 2013).

Clinical studies showed that probiotics have general effects on viral infections of the respiratory tract (**Table 2**). A randomized, double-blind, placebo-controlled study, on preterm infants showed that a synbiotic composed of *L. rhamnosus* GG ATCC5310 and galactooligosaccharides and polydextrose reduced the rate of rhinovirus infection compared to the placebo group (Luoto et al., 2014). In school-aged children, consumption of *L. brevis* KB290 during the influenza season was associated with a reduction in the clinical diagnosis of IFV infection (Waki et al., 2014). In a randomized, double-blind, placebo-controlled pilot study, the probiotic *L. rhamnosus* GG was also associated with a reduction in the occurrence of influenza infections (Wang et al., 2018).

As demonstrated, the most used probiotics in studies of pulmonary diseases caused by viruses are also those of the *Lactobacillus* genus. The effects of this genus related to increased protection against viruses are linked to increased production of IFN types I and II, proinflammatory cytokines such as IL-12 and IFN-γ, or even increased expression of genes encoding anti-viral factors. Unfortunately, a mechanistic basis for the observed beneficial effects of probiotics in combating viral lung infections is often not well defined. This knowledge gap is mainly because most experiments using probiotics for viral treatment use different study designs and experimental models, doses, times, and routes of administration. Therefore, more research is needed to understand better the role of probiotics in our immune system in fighting viral pulmonary infections.

The Gut-Lung Axis During SARS-CoV-2 Infection

The severe acute respiratory syndrome coronavirus 2 (SARS-CIoV-2), which causes coronavirus disease 2019 (COVID-19), has spread around the world since 2019 and has been declared a pandemic that continues to spread with devastating consequences to public health. As of July 2021, there were approximately 190, 600, 300 global confirmed cases and 4,130,000 confirmed deaths.

SARS-CoV-2 can invade human cells by binding its spike protein to a variety of receptors, such as angiotensin-converting enzyme 2 (ACE2), neuropilin-1, tyrosine-protein kinase receptor (AXL), and antibody–FcγR complexes (V'kovski et al., 2021). The current evidence suggests that the severity of COVID-19 is a consequence of a hyperinflammatory immune response culminating in a 'cytokine storm', with markedly increased levels of proinflammatory cytokines such as IL-1, IL-6, IL-12, IFN-γ, and TNF-α, which elicit extensive local and systemic tissue damage (Coperchini et al., 2020).

Recent studies have revealed that patients infected with SARS-CoV-2 demonstrate intestinal dysbiosis, which correlates with the susceptibility and severity of COVID-19 (Zuo et al., 2020; Yeoh et al., 2021). Though some studies detected SARS-CoV-2 RNA in the feces of patients, the activity and infectivity of SARS-CoV-2 in the GI tract are still largely unknown (Zuo et al., 2021). However, as ACE2 is highly expressed in the intestinal epithelia, this receptor may be involved in gastrointestinal symptoms that are common in severe cases (Chen et al., 2020; Villapol, 2020; Koester et al., 2021). Interestingly, in a study with gnotobiotic rats, researchers demonstrated that the gut microbiota regulates the colonic mRNA of ACE2 (Yang et al., 2020).

Another study demonstrated that patients with severe COVID-19 had a significant decrease in the abundance of butyrate-producing bacteria, such as *Faecalibacterium prausnitzii*, *Clostridium butyricum*, *Clostridium leptum*, and *Eubacterium rectale,* and an increased number of common opportunistic pathogens, *Enterococcus* and *Enterobacteriaceae* (Tang et al., 2020). In non-human primates infected with SARS-CoV-2, 16S rRNA analysis of the microbial gut community showed changes in the taxonomic composition, with the relative abundance of *Acinetobacter* and *Ruminococcaceae* being positively correlated with the presence of SARS-CoV-2 in the URT. In addition, SARS-CoV-2 infection significantly alters the metabolite composition with a reduction in the levels of SCFAs, bile acids, and tryptophan metabolites (Sokol et al., 2021).

The impact of probiotics in COVID-19 and in the cytokine storm can be deduced by their known mechanisms in modulating immune response and inflammation (He et al., 2020; de Oliveira et al., 2021), but more basic and clinical research is needed to show their benefits. In a retrospective study of ICU patients diagnosed with pneumonia caused by SARS-CoV-2, the association of the best available therapy with the probiotics *S. thermophilus* DSM 32245, *B. lactis* DSM 32246, *B. lactis* DSM 32247, *L. acidophilus* DSM 32241, *L. helveticus* DSM 32242, *L. paracasei* DSM 32243, *L. plantarum* DSM 32244, and *L. brevis* DSM 27961 showed a positive association with reduced mortality (Ceccarelli et al., 2021).

Gut-Lung Axis Modulation in the Context of Fungal Lung Infections

Among the wide variety of respiratory pathogens, fungi are responsible for only a small proportion of nosocomial or community-acquired pneumonia. However, these species are of relevant medical interest, as fungi cause high morbidity and mortality especially when they affect immunosuppressed patients, or patients with chronic lung diseases, such as chronic obstructive pulmonary disease (COPD) (Charlson et al., 2012; Delhaes et al., 2012).

Although some studies showed that the microbiota in the gut-lung axis is fundamental to the host response to lung infections by fungi, most studies that have demonstrated antimycotic action against respiratory pathogens have been carried out *in vitro*. *In vitro* experiments showed that the bacterium *Bacillus safensis* can block, in a contact-dependent manner, several *Cryptococcus neoformans* virulence factors including melanin, antiphagocytic capsule, and biofilm formation (Mayer and Kronstad, 2017). Many microorganisms of the genus *Bacillus* are characterized as probiotics (Hong et al., 2005); however, *B. safensis* has not yet been characterized as such. *B. safensis* is phylogenetically close to the probiotic *B. pumilus* (Satomi et al., 2006), and its anti-fungal activity has been described (Pandya and Saraf, 2015), making *B. safensis* an exciting candidate for studies of biological safety and probiotic activity. Another study demonstrated *in vitro* that treatment with concentrated cell-free supernatant from the culture of *L. plantarum* 16 altered the transcription of genes involved in a variety of cellular functions, especially those related to cellular metabolism, which culminated in the complete inhibition of spore germination and development of the germ tubes and hyphae of the pathogen *A. fumigatus* (Crowley et al., 2013). In one *in vivo* study using the probiotics *Saccharomyces boulardii*, *L. paracasei* ST-11, and *L. rhamnosus* GG, mice were not protected against lung infection caused by the pathogen *Cryptococcus gattii* (Oliveira et al., 2017).

The recognition of lectins in the fungal cell wall by PRRs is crucial for the activation of dendritic cells and macrophages and the activation of T cells, including Th1 and Th17, which are the best defense strategies against fungal infections, as they help promote the clearance of fungi through innate effectors such as neutrophils and macrophages. The activation of Treg cells and anti-inflammatory cytokines is also fundamental to the anti-fungal immune response, as these cells and molecules are essential for controlling the inflammatory response. The immune response against fungi has already been extensively reviewed by other authors, such as Lionakis et al. (2017).

Among the opportunistic species that affect the lungs, *Aspergillus* spp. are the primary etiologic agents of invasive lung diseases and mainly affect transplant patients (Kontoyiennis et al., 2010; Pappas et al., 2010). Evidence also suggests that COPD patients are at a high risk of developing invasive aspergillosis, although this association is poorly explored (Hammond et al., 2020). An experimental study demonstrated the importance of intestinal microbiota in structuring the pulmonary anti-*Aspergillus* immune response. During infection by *A. fumigatus*, the administration of antibiotics decreased the number of Th17 cells and IL-17 in the lungs, which correlated with a decrease in intestinal colonization by segmented filamentous bacteria (SFB). By investigating how commensal SFBs were linked to this phenotype, the authors, through serum transfer experiments from mice colonized by SFB to negative SFB mice, demonstrated that SFBs contribute to the accumulation of Th17 cells in the lung by inducing an increase in IL-1. This was confirmed when mice that received serum pre-incubated with an IL-1 antagonist attenuated the response of Th17 cells in the lungs (McAleer et al., 2016). Germ-free mice (GF) infected with *C. gattii* showed greater susceptibility to lung colonization, mortality, correlated with reduced levels of IFN-γ, IL-1β, and IL-17 and reduced phagocytosis and ROS production than conventional mice. After restoring the intestinal microbiota those mice mounted a stronger response to infection by *C. gattii*, associated with prolonged survival rates and higher levels of inflammatory mediators (Costa et al., 2016).

Pneumocystis jirovecii is another opportunistic fungus that causes pneumonia, particularly in HIV-positive patients, with an inverse relationship between the $CD4^+$ T cell count in the blood and the risk of infection by *P. jirovecii* (Dunbar et al., 2020). When investigating the diversity of the intestinal microbial community between immunocompetent mice and mice depleted of $CD4^+$ T cells, with pneumonia caused by *Pneumocystis murina*, there was a significant change in alpha and beta diversity and a change in the taxonomic abundance of the intestinal microbiota among these groups, suggesting that the loss of $CD4^+$ T cells affects the intestinal microbiota and the response to *P. murina*. *P. murina* infection was also found to

increase the expression of genes in the intestinal microbiota related to carbohydrate energy metabolism, xenobiotic degradation, and signal transduction pathways (Samuelson et al., 2016a).

Another study demonstrated that vaccination, using a prime-boost vaccination strategy, with live *P. murina* induced protection against subsequent lung infection with the same pathogen in immunocompetent mice and even in mice depleted of $CD4^+$ T cells. In immunocompetent mice, this immunization increased the number of $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells, and $CD11b^+$ macrophages in the lungs after a respiratory infection and increased the levels of IgG and IgA specific for *P. murina*. A significant reduction in the lung load of *P. murina* was observed in serum transfer experiments from non-infected and immunized mice to infected mice. The beta diversity of the intestinal microbial community in mice immunized with *P. murina* was also altered, suggesting that the effectiveness of this immunization may be partly related to the modification of the microbiota; however, further studies are needed to determine whether changes in the microbiota participate in the induction of immunological memory in *P. murina* (Samuelson et al., 2016b).

Despite the importance of the intestinal microbiota and its metabolites for the development of anti-fungal immune responses, and *in vitro* studies demonstrate that probiotics have an action against fungi that cause lung infections, there have been no reports of robust studies aiming to assess the effectiveness of the modulation of *in vivo* intestinal microbiota for the prevention and/or treatment of pulmonary fungal infections. Thus, with the significance of lung infections in mind, more researchers urgently need to turn their attention to this broad and promising field.

Gut-Lung Axis Modulation in the Context of Parasitic Lung Infection

Many helminths cause disease, but they have been shown to also influence the pulmonary immune response. Similar to bacteria, they co-evolved with the host's immune system to maintain a mutually beneficial relationship (Schwartz et al., 2018). Some helminths also share the same niche, the intestinal lumen, with bacteria belonging to the microbiota, and some studies have shown that there are complex interactions between the two (Leung et al., 2018). The intestinal microbiota acts as one of the main inducers of the activation and function of local and systemic antiparasitic responses, such as the activation of Th2 cells and eosinophils (Jiménez-Saiz et al., 2020).

During the larval phase of their life cycle, different species of helminths, such as *Ascaris lumbricoides*, *Toxocara* sp., *Necator americanus*, *Ancylostoma duodenale,* and *Strongyloides* sp., migrate through the lungs and induce pathological immune responses and cause tissue damage, such as eosinophilic pneumonia, which is characterized mainly by increased infiltration of eosinophils in the lung parenchyma and blood eosinophilia (Akuthota and Weller, 2012).

Some studies involving the modulation of the microbiota in spite of helminth infections have focused only on intestinal pathology and on the parasitological aspects of the infection, such as intestinal parasitic load, release of eggs in the feces, and survival of mice. This was demonstrated experimentally with the probiotic *L. casei* ATCC7469 in infection with *Trichinella spiralis* (Bautista-Garfias et al., 2001), *B. animalis* 04450B against infection by *S. venezuelensis* (Oliveira-Sequeira et al., 2014), and *S. boulardii* in mice infected with *T. canis* (Avilada et al., 2012). However, researchers have already reported the beneficial effect of probiotics in reducing the parasitic burden of larval stages during *T. canis* infection in mice. *In vitro* experiments demonstrated a reduction in the viability of *T. canis* larvae after direct incubation with live cells or cell-free supernatant of the probiotic *Enterococcus faecalis* CECT712. The same study also showed that oral administration of *E. faecalis* CECT712 significantly reduced the number of *T. canis* larvae found in the lungs of these animals (Chiodo et al., 2010). *L. rhamnosus* (Walcher et al., 2018) and *L. acidophilus* ATCC 4356 (Cadore et al., 2020) were also able to reduce the parasitic larval burden of *T. canis* in the lungs of mice, but had no antiparasitic action against the larvae *in vitro*, which indicates the indirect action of these two probiotics on *T. canis*, probably related to the stimulation of the protective immune response. The administration of *S. boulardii* in mice infected with *T. canis* increased the transcription of genes encoding IL-12 and IFNγ, which correlated with a decrease in the number of larvae in the lungs and other tissues (de Avila et al., 2016).

Some probiotics, mainly from the *Lactobacillus* genera, showed action *in vitro* and *in vivo* against helminths that cycle through the lungs. However, differences in the efficacy of species and strains used can be attributed to variability in the experimental models, the probiotic dose, and the administration route. However, data are insufficient to determine the molecular mechanisms by which probiotics act on helminths that cycle through the lungs. Furthermore, further studies on host-microbiota-helminth interaction mechanisms are needed to validate the actions of probiotics in clinical studies with humans.

STUDIES PERSPECTIVES AND CONCLUSION

Although studies have shown that probiotics, prebiotics, and synbiotics have prophylactic and therapeutic effects against lung infections caused by bacteria, viruses, fungi, and helminths, further studies are needed to better understand the mechanisms of action and molecular pathways involved in these strategies. It is necessary to favor the translational use of gut microbiota modulation strategies as a therapeutic approach to human lung diseases. In addition, since the effect of these strategies is highly linked to the strains of the microorganism and the dose and route of administration, more in-depth investigations should be performed, considering well-defined experimental protocols. Considering the complexity of the microbiota and its interaction with the host, it is also important to determine whether this strategy acts in synergy with the microbiota or has another mechanism involving direct action against the lung pathogen or modulation of the host

immune system. In addition, it is important to emphasize the therapeutic window necessary to restore the gut microbiota to re-establish homeostasis after lung infection control.

AUTHOR CONTRIBUTIONS

CC wrote the manuscript, tables, and made the figures; MR and AV contributed to the design, writing, and review of the manuscript. The authors read and approved the final manuscript.

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Probiotics-Based Treatment as an Integral Approach for Alcohol Use Disorder in Alcoholic Liver Disease

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Alcoholic liver disease (ALD) is one of the leading causes of morbidity among adults with alcohol use disorder (AUD) worldwide. Its clinical course ranges from steatosis to alcoholic hepatitis, progressing to more severe forms of liver damage, such as cirrhosis and hepatocellular carcinoma. The pathogenesis of ALD is complex and diverse elements are involved in its development, including environmental factors, genetic predisposition, the immune response, and the gut-liver axis interaction. Chronic alcohol consumption induces changes in gut microbiota that are associated with a loss of intestinal barrier function and inflammatory responses which reinforce a liver damage progression triggered by alcohol. Alcohol metabolites such as acetaldehyde, lipid peroxidation-derived aldehyde malondialdehyde (MDA), and protein-adducts act as liver-damaging hepatotoxins and potentiate systemic inflammation. Additionally, ethanol causes direct damage to the central nervous system (CNS) by crossing the blood-brain barrier (BBB), provoking oxidative stress contributing to neuroinflammation. Overall, these processes have been associated with susceptibility to depression, anxiety, and alcohol craving in ALD. Recent evidence has shown that probiotics can reverse alcohol-induced changes of the microbiota and prevent ALD progression by restoring gut microbial composition. However, the impact of probiotics on alcohol consumption behavior has been less explored. Probiotics have been used to treat various conditions by restoring microbiota and decreasing systemic and CNS inflammation. The results of some studies suggest that probiotics might improve mental function in Alzheimer's, autism spectrum disorder, and attenuated morphine analgesic tolerance. In this sense, it has been observed that gut microbiota composition alterations, as well as its modulation using probiotics, elicit changes in neurotransmitter signals in the brain, especially in the dopamine reward circuit. Consequently, it is not difficult to imagine that a probiotics-based complementary treatment to ALD might reduce disease progression mediated by lower alcohol consumption. This review aims to present an update of the pathophysiologic mechanism underlying the microbiota-gut-liver-brain axis in ALD, as well as to provide evidence supporting probiotic use as a complementary therapy to address alcohol consumption disorder and its consequences on liver damage.

Keywords: alcoholic liver disease, microbiota, gut-liver-brain axis, probiotics, alcohol craving, alcohol addiction, neuroinflammation

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INTRODUCTION

Alcohol consumption is the third most important cause of health impairment worldwide, with 5.3% of all annual deaths due to its excessive use. Approximately 43% of the population over 15 years of age consumed alcohol in the last 12 months, indicating an early life risk of death and disability due to this cause (World-Health-Organization, 2018).

Chronic alcohol consumption is one of the main risk factors of liver injury (Rocco et al., 2014), with alcoholic liver disease (ALD) as one of the leading causes of morbidity among adults with alcohol use disorder (AUD). The liver damage induced by alcohol consumption includes the following clinical impacts: steatosis, steatohepatitis, alcoholic hepatitis, fibrosis, and cirrhosis, each considered a relevant public health burden (World-Health-Organization, 2018). Globally, AUD has a significant socioeconomic impact on the population, with an elevated mortality rate from alcohol cirrhosis associated with increased alcohol consumption rates. It is estimated that alcohol consumption and ALD incidence will continue to increase in the coming decades, inextricably linked to psychosocial issues that our society is facing.

Consequently, healthcare systems confront a significant and increasing demand for ALD treatment. So far, abstinence-based interventions remain the cornerstone of clinical ALD management. However, due to the high relapse rate observed in AUD patients, there are increasing needs for developing and implementing new treatment options for this disorder (Axley et al., 2019).

In recent years, numerous studies have focused on the role of the microbiota-gut-liver axis in ALD pathophysiology. Diverse strategies directed to reestablish the homeostatic function of this axis have also been assayed in ALD patients with successful therapeutical results, including probiotic-based approaches. In this review, we summarize some of this evidence, including an additional landscape focused on integrating this knowledge to the role of the brain functions over these mechanisms and vice-versa. Bidirectional modulation of this relationship will help advance toward better integral management of this pathology, which is based on the microbiota-gut-liver-brain axis as a central component in ALD.

MICROBIOTA-GUT-LIVER AXIS IN THE PATHOGENESIS OF ALCOHOLIC LIVER DISEASE

Once a drink is swallowed, it is mainly absorbed in the intestinal tract and subsequently transported *via* the portal vein to the liver, where it is metabolized. A significant part of absorbed alcohol can induce direct damage to this organ. However, only 10–35% of heavy drinkers develop alcoholic steatohepatitis, and of those subjects, 10% develop liver cirrhosis (McCullough and O'Connor, 1998), suggesting that other mechanisms can contribute to the ALD pathogenesis.

ALD pathogenesis is complex and multifactorial, including environmental factors, genetic predisposition, immune response, and gut microbiota. In recent years, several researchers have focused on studying ALD pathogenesis regarding the interaction between the gut microbiota and the liver. The influence of intestinal microbiota on liver disease development has been highlighted among the findings, as well as, contrariwise, the impact exerted by the liver and bile acid secretion on microbiota status (Szabo, 2015). In this regard, abusive alcohol consumption influences the microbiota-gut-liver axis interaction, a mechanism highly relevant to ALD progression (Bajaj, 2019). The interplay of the components belonging to the axis sets the behavior of diverse mechanisms that are part of it, such as intestinal immune responses, intestinal barrier function, microbiota composition, and hepatic and systemic inflammation, all of which are seriously altered in ALD (Leclercq et al., 2014b; Chen et al., 2015; Neuman et al., 2020).

Increasing evidence has demonstrated that alcohol intake leads to small and large intestinal changes in intestinal microbial composition and a loss of intestinal barrier function, giving rise to an inflammatory response that reinforces the liver damage progression triggered by alcohol. Differences in microbiota diversity and composition have been described in the pathophysiology of many diseases such as Inflammatory Bowel Disease, Parkinson's, and Autism (Bajaj, 2019). A particular dysbiosis is observed for ALD, which is described to be conservative across the studied populations and closely associated with the severity of alcohol dependence (Llopis et al., 2016). Compared to healthy subjects, the dysbiosis observed in AUD is characterized by decreased abundance for the phylum *Bacteroidetes* but elevated for *Proteobacteria*, while at the family level, an increased number of Enterobacteriaceae has been observed in individuals with cirrhosis, which is related to plasma endotoxin abundance increases. By contrast, *Lachnospiracea* and Ruminococcaceae have lower abundance in individuals with AUD, which is linked with reduced intestinal short-chain fatty acids (SCFAs) (Litwinowicz et al., 2020). Since SCFAs are products derived from bacterial fermentation, changes in intestinal microbial composition might be related to differences in intestinal metabolism responsible for decreased SCFA levels observed after alcohol intake (Hartmann et al., 2015). SCFAs provide energy to enterocytes and exert a protective effect on the gut barrier function by promoting an anti-inflammatory environment, thus mediated by regulatory mechanisms of immune response activation (Litwinowicz et al., 2020). Additionally, at the genus level, increased levels of *Bifidobacterium* and *Streptococcus* have been shown after alcohol consumption, being described as the most common pathogens responsible for bacterial infections in cirrhotic individuals (Litwinowicz et al., 2020). In this context, Zhong X. et al. demonstrated that increased *Streptococcus* abundance was linked with hepatocyte damage severity in patients with alcoholic liver cirrhosis, which in turn was correlated with AST plasma level, a major indicator of alcoholic liver injury (Zhong et al., 2021).

The factors contributing to dysbiosis in ALD are not fully known. However, it has been described that environmental factors, genetics, intestinal dysmotility, increased gastric pH, altered bile flow, and an altered immune response participate

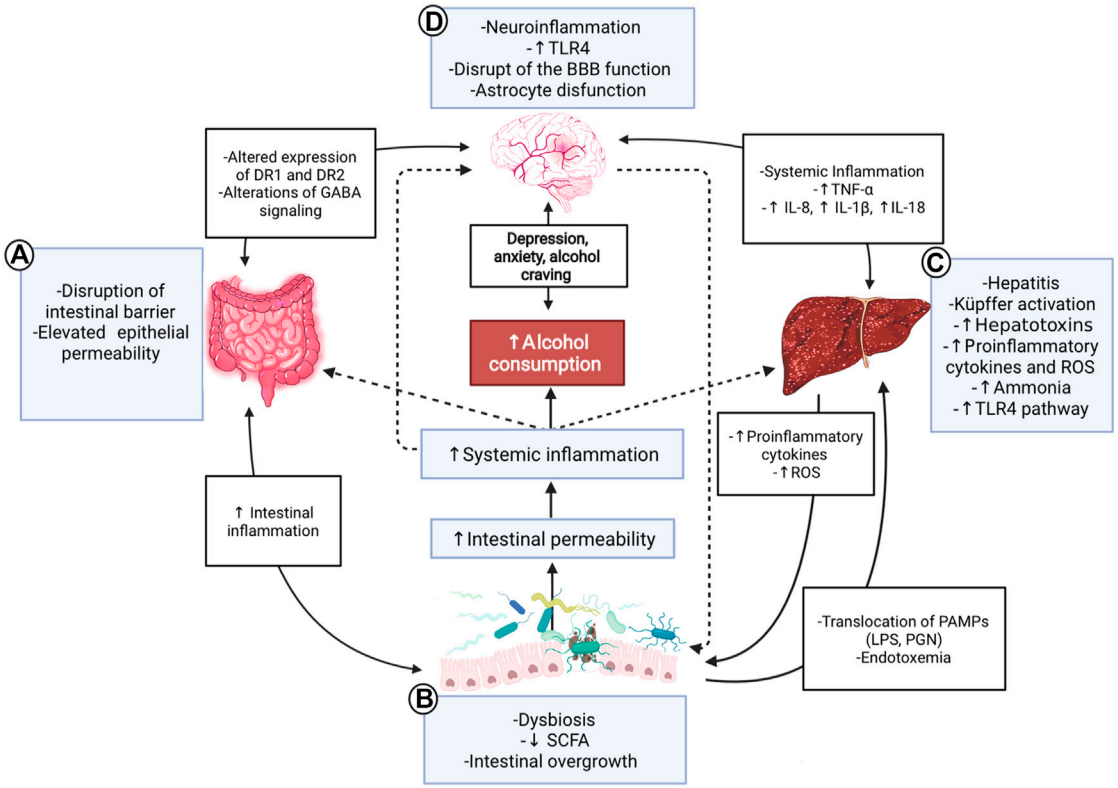

FIGURE 1 | Gut-microbiota-liver-brain axis in ALD. Interaction diagram of the different mechanisms participating in the gut-microbiota-liver-brain axis involved in the pathophysiology of ALD. **(A)** Alcohol consumption has adverse effects on the gut; it disrupts the gut barrier leading to high permeability and translocation of bacterial products. These effects create a proinflammatory environment which affects microbiota. **(B)** ALD has a specific microbiota dysbiosis favoring an overgrowth of nonbeneficial bacteria. The decrease of SCFA due to alcohol consumption influences these alterations because SCFA is food for helpful bacteria. This context produces a translocation of different substances called PAMPs, such as LPS or peptidoglycan, to the liver and circulation, increasing endotoxemia. **(C)** The liver is a vital organ in ethanol metabolization and suffers many changes in chronic consumption; activation of Küpffer cells and proinflammatory TLR4 pathway, causing hepatitis, increased reactive oxygen species, and cytokines, such as IL-18, IL-8, and IL-1β. In advanced stages, the liver fails in its detox task, and organisms accumulate ammonia. **(D)** All the aforementioned inflammatory processes lead to a systemic inflammation that affects the brain, contributing to ethanol-triggered neuroinflammation. PAMPs and alcohol also produce disruption of the blood-brain barrier, astrocyte senescence, and more significant changes in the brain; alteration of the DR1 and 2, increased levels of anxiety, depression, and alcohol craving. Finally, the gut and the microbiota are influenced by the brain and vice-versa through nerve and GABA signaling modulation. ALD: Alcoholic liver disease; SCFA: Short-chain fatty acids; PAMPs: Pathogen-associated molecular patterns; LPS: Lipopolysaccharide: PGN: Peptidoglycan; ROS: Reactive oxygen species; BBB: Blood-brain barrier; DR1/DR2: Dopamine receptor 1/2; GABA: γ-aminobutyric acid; TLR4: Toll-like receptor 4.

in its development (Hartmann et al., 2015). Moreover, the down-regulation of intestinal antimicrobial peptides (AMPs) after chronic ethanol consumption (Litwinowicz et al., 2020) contributes to intestinal dysbiosis. Intestinal alpha-defensins are AMPs that play an innate host defense against bacterial infection and maintain intestinal mucosa homeostasis (Muniz et al., 2012). It has been shown that chronic ethanol intake down-regulates the expression of alpha-defensins in the intestine, leading to dysbiosis, loss of intestinal barrier function, and systemic inflammation (Shukla et al., 2018). In this regard, new evidence has shown that cathelicidin-related antimicrobial peptide (CRAMP) knockout mice fed with alcohol exacerbate ALD response by an increased hepatic inflammasome activation and an elevated serum interleukin (IL)-1β levels. Indeed, the exogenous administration of CRAMP can reduce alcohol-induced hepatic steatosis by reverting alcohol-induced endotoxemia and inflammasome activation (Li et al., 2020).

Chronic alcohol ingestion also may lead to small and large intestinal bacterial overgrowth, which along with changes in the microbiota composition, have been correlated with alcoholic cirrhosis severity. This evidence suggests that microbiota modulation can be an attractive target for ALD therapy (Hartmann et al., 2015). Dysbiosis in ALD led to an abnormal accumulation of bacterial products in the portal circulation (Tilg et al., 2016). In fact, dysbiosis, bacterial overgrowth, and alcohol consumption are associated with increased intestinal epithelial permeability, facilitating microbial product's translocation to the liver, including lipopolysaccharide (LPS), an endotoxin from Gram-negative bacteria (**Figure 1**) (Araneda et al., 2016). Several studies have demonstrated that alcohol consumption increases LPS levels in the systemic circulation, mainly observed during the early stages of ALD. Upon reaching the liver, LPS activates inflammatory pathways conducted by interacting with Toll-like receptor-4 (TLR-4), triggering

intracellular signaling, principally regulated by the nuclear factor-kappa B (NF-kB), toward the expression of the inflammatory genes. Consequently, the release of proinflammatory cytokines by Küpffer and other hepatic cells occurs, inducing liver and systemic inflammation (Hartmann et al., 2015; Araneda et al., 2016). Among the cytokines TNF-α stands out as a proinflammatory cytokine that induces liver fibrosis and necro-inflammatory hepatic damage processes. High systemic TNF-α levels are also associated with worsening gut permeability (Rocco et al., 2014) and intestinal inflammatory responses that enlarge the initial impact induced by alcohol over the gut microbiota composition.

The liver is the main organ responsible for ethanol metabolism. Ethanol oxidation can occur in two steps: the first is conducted by alcohol dehydrogenase (ADH), a cytoplasmic enzyme promoting fast oxidation from ethanol to acetaldehyde, a process that occurs mainly in the liver due to a high expression of the enzyme in this organ (Seitz and Oneta, 1998). ADH expression is also observed in the gut, associated with a lesser degree of alcohol metabolism, limiting the ethanol charge in the portal vein and, thus, in the liver and the systemic circulation (Seitz et al., 1994). Subsequently, acetaldehyde is further metabolized to acetate in a second stage by acetaldehyde dehydrogenase (ALDH).

Ethanol and its metabolites can exert a direct cytotoxic effect on the cells acting as hepatotoxins. Acetaldehyde damages the liver by triggering inflammation, extracellular matrix remodeling, and fibrogenesis (Rocco et al., 2014). Additionally, acetaldehyde can directly disrupt the epithelial barrier function. *In vitro* studies conducted by K. J. Atkinson and R. K. Rao showed that acetaldehyde, at elevated pathophysiological concentrations, was able to disrupt tight junction structures of Caco-2 cell monolayers, mainly zonula occludens-1, by a tyrosine phosphorylation-dependent mechanism, contributing to increased gut permeability (Atkinson and Rao, 2001).

ADH conducts the main route to metabolize ethanol. However, chronic alcohol consumption upregulated the microsomal ethanol oxidizing system by cytochrome P450 (CYP) enzymes, specifically CYP 2E1. First, CYP 2E1 catalyzes ethanol oxidation to acetaldehyde and then metabolizes it to acetate (Ceni et al., 2014). The catalytic reaction of ethanol by CYP2E1 generates significant reactive oxygen species, such as superoxide anion, hydrogen peroxide, and the hydroxyl radical. These molecules can induce direct damage to hepatic cells, generating toxic effects such as lipid peroxidation, enzyme inactivation, DNA mutations, and cell membrane destruction (Ceni et al., 2014). Reactive oxygen species can also induce inflammatory processes of alcohol-induced liver damage by recruiting immune cells to the liver, increasing systemic proinflammatory cytokine levels, and contributing to lipid peroxidation (Rocco et al., 2014). Lipid peroxidation is one of the main reactions in alcohol-induced liver damage due to the generation of toxic aldehydes, including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). Similar to acetaldehyde, these molecules can react with DNA, lipids, and proteins to form adducts (Ceni et al., 2014; Rocco et al., 2014) that interfere with liver function by mechanisms of mitochondrial damage, activation of stellate cells, increased liver fibrosis, and inflammation (Ceni et al., 2014).

The mechanisms involved in the communication of the microbiota-gut-liver axis that continuously contributes to ALD development are not alone. The reciprocal impact of brain function perturbations in ALD progression has acquired increasing importance.

ALCOHOL AND MICROBIOTA-GUT-LIVER-BRAIN AXIS

The alterations of the microbiota-gut-liver axis in ALD have been widely described during the last years. Interest has recently increased regarding the role of this axis in brain function and its reciprocal influence on the intestinal environment and liver functions. Thus, growing evidence has emerged to consider the microbiota-gut-liver-brain axis as an integrative approach for better understanding ALD pathophysiology.

As mentioned earlier, diverse evidence has shown that microbiota disturbances and liver damage affect gut-brain axis communication. In this regard, Stärkel P. et al. observed that depression, anxiety, and alcohol craving are positively correlated with increased intestinal permeability in patients with alcohol dependence (Leclercq et al., 2014a). Moreover, brain function alteration in primary psychiatric disorders such as schizophrenia, in the absence of AUDs, is associated with gut-brain axis interaction disturbances that are enhanced by alcohol consumption (Bajaj, 2019).

Brain function is affected throughout the spectrum of AUDs, ranging from acute intoxication to chronic changes, such as hepatic encephalopathy (Bajaj, 2019). The direct effects of alcohol on the brain are explained because ethanol is a lipophilic molecule that easily crosses the blood-brain barrier, causing direct damage to the central nervous system (CNS). Among its deleterious effects is increased neuronal membrane fluidity, which can be mediated by lipid composition proportion changes (Leonard, 1986) and genotoxic damage that leads to cell death (Lamarche et al., 2003). In addition, endogenous DNA-damaging molecules, such as oxygen radicals, lipid peroxidation products, and acetaldehyde, all produced due to ethanol metabolism, contribute to this process (Brooks, 1997). Ethanol also activates an immune response in the brain conducted by an increased TLR4 pathway activation. It consequently induces inflammatory cytokines, such as TNF-α and IL-6, mediating neuroinflammation and blood-brain barrier impairment (Gupta et al., 2021). Inflammatory brain damage contributes to alcohol dependence after its chronic and heavy consumption. Furthermore, brain reward circuit activation enhances this behavior, which is associated with a positive reinforcement that drinking exerts on further ethanol intake, due partially to dopamine production (Stärkel et al., 2016).

As we mentioned earlier, the impact of alcohol on brain functions can indirectly be mediated by gut-liver-brain axis disturbance. Alcohol-induced microbiota changes and its consequences on intestinal barrier function can contribute to bacterial components and metabolites translocating to the

bloodstream and liver, inducing low-grade systemic inflammation. In this regard, increased bacteria component loads in peripheral circulation have also been associated with alcohol dependence and consumption habits (Leclercq et al., 2012; Stärkel et al., 2016). This generates a vicious circle, where alcohol-induced microbiota damage leads to consuming more alcohol, and its ingestion perpetuates the intestinal microenvironment injury. In this regard, Jadhav KS. et al. demonstrated that a differential microbiota composition was associated with alcohol consumption behavior in vulnerable and resilient experimental rat groups trained daily to self-drink 0.1 ml of alcohol (10% weight/volume) during 80 following sessions of 30 min. They observed that, unlike a resilient group of rats, the vulnerable group (those that lose control over alcohol consumption) showed microbiota composition changes and were correlated with striatal dopamine receptor expression level alterations (Jadhav et al., 2018). These results suggest a regulatory role of microbiota over the dopamine reward system in the brain.

The mesocorticolimbic dopamine system or reward system consists of heterogeneous dopaminergic neurons localized in the mesencephalon, diencephalon, and olfactory bulb. Mesodiencephalic dopaminergic neurons are part of substantia nigra pars compacta, the ventral tegmental area (VTA), and the retrorubral field. The dopamine system includes the mesolimbic and mesocortical pathways, which arise from VTA. The mesolimbic dopaminergic system includes VTA that project to the nucleus accumbens, amygdala, and hippocampus. The mesocortical dopaminergic system, which includes the VTA, extends its fibers to the prefrontal, cingulate, and perirhinal cortex (Arias-Carrión et al., 2010). As a component of the reward pathway, the striatum comprises medium spiny neurons classified into those expressing dopamine receptor D1, the direct pathway, and those expressing the D2 receptor or indirect pathway as a reward pathway component. D1 medium spiny neurons mediate reinforcement and reward, so a current consensus suggests that D1 medium spiny neurons facilitate the selection of rewarding actions. D2 medium spiny neurons, by contrast, have been associated with aversion and avoidance, so D2 medium spiny neurons help suppress cortical patterns that encode maladaptive or non-rewarding actions (Jadhav et al., 2018). Therefore, positive reinforcement learning would be modulated by signaling the D1 direct pathway, while negative reinforcement learning would be modulated by signaling the D2 indirect pathway (Jadhav et al., 2018). In the Jadhav KS study, the vulnerable group of rats showed a lower expression of striatal D2 receptors, concomitant with higher expression of D1 receptors at the striatum. These findings suggest that dysbiosis-induced alcohol consumption predisposition was due to a higher reward effect.

Regarding the study, an interesting association between D2R mRNA expression and microbiota composition was described in the vulnerable group. A significant correlation was found between changes in the low abundance of some bacteria genera, such as Lachnospiraceae, and reduced D2R mRNA expression in the brain. These findings have suggested that reestablishing gut microbiota composition may contribute to inhibitory innervations in brain circuits associated with addiction. The correlations between intestinal microbial composition and addiction behavior would indicate that variations in bacterial abundance may coincide with differences in the addictive behavior, connecting the gut microbiota and the brain directly, specifically to the striatal D2R mRNA expression (Jadhav et al., 2018).

As we already mentioned, the liver damage stage is linked with intestinal dysbiosis progression. Concurrently, this is associated with increased intestinal permeability and microbial product translocation to the liver, promoting bile acid metabolism imbalance, gut dysmotility, and systemic inflammation (Milosevic et al., 2019). Ammonia and other substances produced by the intestinal microbiota that are cleared by the liver can also be accumulated in ALD. Consequently, high circulating ammonia levels reaching the CNS induce astrocyte senescence, giving rise to a cascade of events leading to brain damage (Gupta et al., 2021). Brain imaging studies have demonstrated that hyperammonemia is related to astrocyte dysfunction (Ahluwalia et al., 2016). Furthermore, an increased level of proinflammatory plasma cytokines, such as TNF-α, also contributes to this inflammatory brain damage (Gupta et al., 2021). Therefore, microbial products, ammonia, and inflammatory mediators produced by disturbances of the microbiota-gut-liver axis can worsen the neuroinflammation of the brain in ALD.

Neurobiological Alteration in Alcohol Addiction and Neuroinflammation

As previously mentioned, ALD is directly associated with the damage produced by alcohol consumption, making it important to go further into the subject of alcohol addiction and the mechanisms involved in its pathogenesis. Recent studies have been focused on how an imbalance in the microbiota-gut-liver-brain axis, due to alcohol consumption, affects brain function in people with ALD, specifically in their cognitive performance (Ahluwalia et al., 2016). Alcohol impacts multiple brain pathways, neuroplasticity, signaling related to reward, stress, habit formation, and decision making, which contribute to producing the phenomenon of addiction (Koob and Volkow, 2010). However, the exact mechanisms exerted by alcohol on the brain and the association between alcohol addiction and the microbiota-gut-liver-brain axis are still unknown.

Chronic administration of alcohol and other abused substances activates the mesocorticolimbic dopamine system, producing functional alterations at several levels (Adinoff, 2004). Ethanol is known to provoke a dose-dependent excitation of dopaminergic VTA neurons (Brodie et al., 1990), increasing dopamine levels in the nucleus accumbens. This finding is relevant, considering that in the pathophysiology of addiction, dopamine synapse plasticity and metaplasticity play an important role in reward-based learning and addiction development (Cui et al., 2013). Interestingly, new evidence suggests that self-administration of ethanol is not dependent only on the dopaminergic activation of the nucleus accumbens. Indeed, this event is necessary for rewarding the

effects of ethanol but not essential for other aspects of reinforcing actions of the drug (Weiss and Porrino, 2002).

In this regard, other neuronal pathways contribute to the development of alcohol addiction. It has been demonstrated that ethanol can directly interact with $GABA_A$ and NMDA ion channel receptors in the mesocortical system by an unknown mechanism. This interaction mediates the reinforcing action of alcohol. Chronic intake and repeated ethanol withdrawal experiences produce $GABA_A$ receptor function adaptations, decreasing its sensitivity. As with inhibitory neurotransmission signaling in the CNS, an increased GABAergic activation by ethanol is related to decreased neuronal excitability in diverse brain areas, including the prefrontal cortex area (Grobin et al., 1998). Therefore, the adaptations induced by ethanol are important in the marked increased CNS excitability that characterizes the withdrawal (Finn and Crabbe, 1997).

Conversely, glutamate is the principal excitatory neurotransmitter in the brain. Ethanol plays a role in modulating ionotropic glutamate receptors, with NMDA receptors being the most studied. Chronic alcohol consumption causes an adaptive up-regulation of the NMDA receptor function (Hoffman and Tabakoff, 1994), a mechanism that could explain withdrawal symptoms that appear due to rebound activation of this receptor.

Another neural signaling pathway involved in alcohol addiction is serotonergic system dysfunction. In abstinent alcoholics, a decreased serotonin (5-HT) content is observed in cerebrospinal fluid, platelet, and low use of tryptophan, the amino acid precursor of serotonin. In line with this evidence, various studies have observed a decrease in plasma tryptophan concentrations in alcohol-dependent patients. Tryptophan deposit depletion in alcoholics does not increase alcohol consumption behavior (Sari et al., 2011). Studies carried out in humans regarding the administration of central serotonergic agonists have not yet provided concordant results, but a significant reduction in the availability of brainstem serotonin transporters was found in alcoholics, which was correlated with alcohol consumption, depression, and anxiety during withdrawal. These findings support the hypothesis of serotonergic dysfunction in alcoholism (Heinz, 1998).

New evidence has suggested that cerebral neuroimmune interaction also plays a role in addiction. Neuroimmune mediators expressed in neurons and glia, such as cytokines and chemokines, are involved in various brain functions. For instance, it has been described that CCL2 and CXCL-12 regulate the release of glutamate, GABA, and dopamine (Cui et al., 2014). Neurotransmitters are involved in the reward system. These findings open new opportunities for exploring the role of this neuroimmune communication in alcohol addiction.

Neuroinflammation involves diverse stages. Initially, an innate immune response, principally characterized by increased levels of TNF-α and IL-1β, is produced by microglia in response to environmental toxins or neuronal damage. These cytokines exert neuroprotective effects on SNC by promoting oligodendrocyte maturation and neurotrophin secretion. However, under overactivated conditions, microglia release abundant proinflammatory cytokines and chemokines, which synergistically mediate neuroinflammatory processes in specific brain areas, such as the central amygdala (Cui et al., 2014). *In vivo* animal studies provide further evidence about the role of neuroimmune modulation in alcohol addiction; some studies show effects from interrupting certain neuroimmune gene expressions, such as beta-2-microglobulin and cathepsin S (Blednov et al., 2005; Blednov et al., 2012) or targeted disruption of TLR4 in the central amygdala reduced alcohol consumption (Liu et al., 2011). Indeed, pharmacological suppression of neuroimmune signaling pathways, such as the toll-like receptor signaling pathway, reduces alcohol intake behavior in different animal models (Mayfield et al., 2013; Bell et al., 2015). In this regard, alcoholics have shown a positive correlation between alcohol craving and elevated levels of inflammatory cytokines and endotoxins in serum, suggesting that an innate immunity activation may uphold alcohol addiction. This premise is consistent with results obtained from animal studies where injecting LPS increased alcohol consumption, with this effect reversed by deleting immune-related genes (Cui et al., 2014). In this scenario, it is not difficult to imagine that, by an indirect effect of probiotics on microbiota modulation and the reduction of systemic inflammation, they could be a good therapeutic alternative to control alcohol addiction. Probiotic's impact on alcohol-neuroinflammation has been poorly explored. Further studies directed to understand the role of probiotics in cerebral neuroimmune alterations are necessary to comprehend its contribution to alcohol addiction.

While chronic alcohol consumption induces neuroinflammation in the CNS, the peripheral elevation of cytokine levels can promote and reinforce this damaging process. Systemic inflammation is favored by the activation conducted by pathogen-associated molecular patterns (PAMPs), such as LPS and peptidoglycan, over Pattern Recognition Receptors (PRRs) (TLRs or NOD-like receptors) present in various immune cells. It has been seen that the activation of this pathway plays a crucial role in developing alcohol-induced damage, given that they trigger the expression of genes involved in the innate immune response. Thus, the elevation of proinflammatory cytokine levels, such as IL-1β, IL-8, and IL-18 (Akira et al., 2006; Leclercq et al., 2014a) results in a systemic and SNC low-grade inflammation. The contribution of this mechanism in ALD pathogenesis has been strongly demonstrated in TLR4 knockout mice experiments characterized by acquired resistance to both alcohol addiction and liver-damaging (Alfonso-Loeches et al., 2010). Furthermore, these proinflammatory pathways have been directly related to a greater desire for alcohol consumption or craving, as well as its dependence and addiction (Leclercq et al., 2012).

ALD is the most common cause of death among patients with AUD and is considered a preventable disorder. Currently, the alternatives for AUD treatment are limited, including psychological and pharmacological therapy characterized by low efficacy. Some drugs approved by the Food and Drug Administration (FDA), such as disulfiram, naltrexone, and acamprosate, are currently being used to reduce feel-good response to alcohol intake and control the long-term effect of

alcohol deprivation (Vuittonet et al., 2014). However, other unapproved drugs, such as gabapentin, baclofen, topiramate, ondansetron, varenicline, and other approved drugs such as nalmefene, are beginning to be used off-label (Leggio and Lee, 2017; Shen, 2018). Therefore, there is a need for a treatment that supports current therapies. In addition, recent studies have positioned ethanol-induced neuroinflammation as a central factor in alcohol dependence and depressive and anxiety disorders, with the latter also present in conjunction with AUD, probably because they share the same pathophysiology.

As previously discussed, alcohol leads to CNS and systemic inflammation both directly and indirectly and is a relevant factor to understand while seeking alcohol addiction treatments. The low-grade inflammation observed in AUD is associated with mood disorders, mental illness (schizophrenia and autism), and alcohol addiction, which can develop together. Indeed, depression predisposes to alcoholism and vice versa (Maes, 1999; Felger and Lotrich, 2013). Likewise, the presence of depression symptoms has been widely studied in patients with chronic inflammation. It is shown that the increase in inflammatory cytokines, such as interferon-γ, generates a greater expression of enzymes involved in tryptophan catabolism, the precursor of serotonin. Thus, the persistence of a low-grade inflammation status would explain the appearance of mood disorders frequently observed in alcohol consumption (Lestage et al., 2002). Considering the aforementioned mechanisms, it is not difficult to imagine that reducing systemic inflammation would reduce detrimental psychological symptoms and addiction behavior to alcohol and other drugs, such as cocaine and opioids (Koob and Le Moal, 2005; Zhang et al., 2019).

Given the addictive nature of alcohol, strategies to prevent relapse after withdrawal are currently being investigated. Several studies suggest that intestinal microbiota modulation using probiotics may have a role in ALD. Reestablishing beneficial gut bacteria composition would decrease anxiety, depression, and neuroinflammation in AUD patients and decrease alcohol consumption. Therefore, complementary therapies based on probiotics are an attractive therapeutic alternative to treat addictions and their relapses.

PROBIOTICS AND GUT-BASED THERAPY

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (Mack, 2005). Probiotics' beneficial effects have been widely studied in different pathologies, such as gastrointestinal diseases, and to treat various central disorders by restoring microbiota properties and the capability to modulate systemic and CNS inflammation. Furthermore, due to probiotics' potential benefits for CNS and mental disorders, it has recently been proposed to recognize them as "psychobiotic," with an expectation of low side effects and anti-inflammatory, antidepressant, and anti-anxiety properties (Ansari et al., 2020). Some studies suggest using probiotics to improve mental function in Alzheimer's and Autism spectrum disorders and attenuated morphine-derived analgesic tolerance (Zhang et al., 2019; Ansari et al., 2020).

Probiotics Benefits on Alcoholic Liver Disease

Diverse studies have shown that probiotics have beneficial effects on ALD. Probiotics can modulate several pathophysiological mechanisms involved in liver damage development, some of them detailed in **Figure 2**. Among the mechanisms described are microbiota balance restoration, decreasing dysbiosis, and promoting an anti-inflammatory environment that allows for reducing intestinal permeability and translocating of bacterial components (LPS) to the systemic circulation (Kirpich et al., 2008). In addition, by reducing endotoxemia, probiotics can prevent bacterial metabolites reaching the liver and the inflammatory response (Kirpich et al., 2008).

As previously mentioned, changes in the microbiota-gut-liver-brain axis are observed for diverse behavioral and addictive disorders. It is not difficult to imagine that the use of approaches directed to modulate these alterations may treat AUDs. A positive mechanism of probiotics could thus have different consequences in ALD development (**Figure 2**). It is therefore simple to conceive the possibility of addressing alcohol addiction with probiotics to positively reduce depression, anxiety, alcohol craving, dependence, and systemic inflammation. By reducing systemic proinflammatory status and neuroinflammation, probiotics also offer an excellent alternative to relieve CNS damage reinforcing beneficial effects on addiction and, consequently, alcohol consumption.

Numerous approaches have been explored to modulate intestinal microbiota in ALD. **Table 1** summarizes some of the studies related to probiotics use for modulating mechanisms underlying the microbiota-gut-liver-brain axis in this disorder. One highlight is the study of Kirpich IA. et al. who observed that probiotic supplementation with *Bifidobacterium bifidum* and *Lactobacillus plantarum* restored Bifidobacteria, Lactobacilli, and Enterococci numbers in a group of alcoholics, to the title reported in healthy controls (Kirpich et al., 2008). Some probiotics approaches have also been shown to modulate intestinal barrier function in AUD patients, mainly using *Lactobacillus*, *Bifidobacterium bifidum*, or *Akkermansia* (Khailova et al., 2009; Grander et al., 2018). In this regard, other probiotics have been reported to improve the expression of tight junction proteins in the ileum and normalize cytokine levels (Chen et al., 2016) in mice with a chronic binge alcohol-fed model. Other studies have revealed that probiotics have beneficial effects on brain functions, mood, behavior, and addiction. For example, a recent study has shown that in a group of patients with alcoholic hepatitis treated orally with cultures of *Lactobacillus subtilis* and *Streptococcus faecium,* the probiotic-based treatment decreased serum LPS level compared with placebo (Han et al., 2015). This study also shows that probiotic-based therapy modulates the microbiota environment, specifically reducing *E. coli* levels and increasing *Lactobacillus* in patients with alcoholic hepatitis. Interestingly, a considerably decreased LPS level was observed in a subgroup of patients with high liver

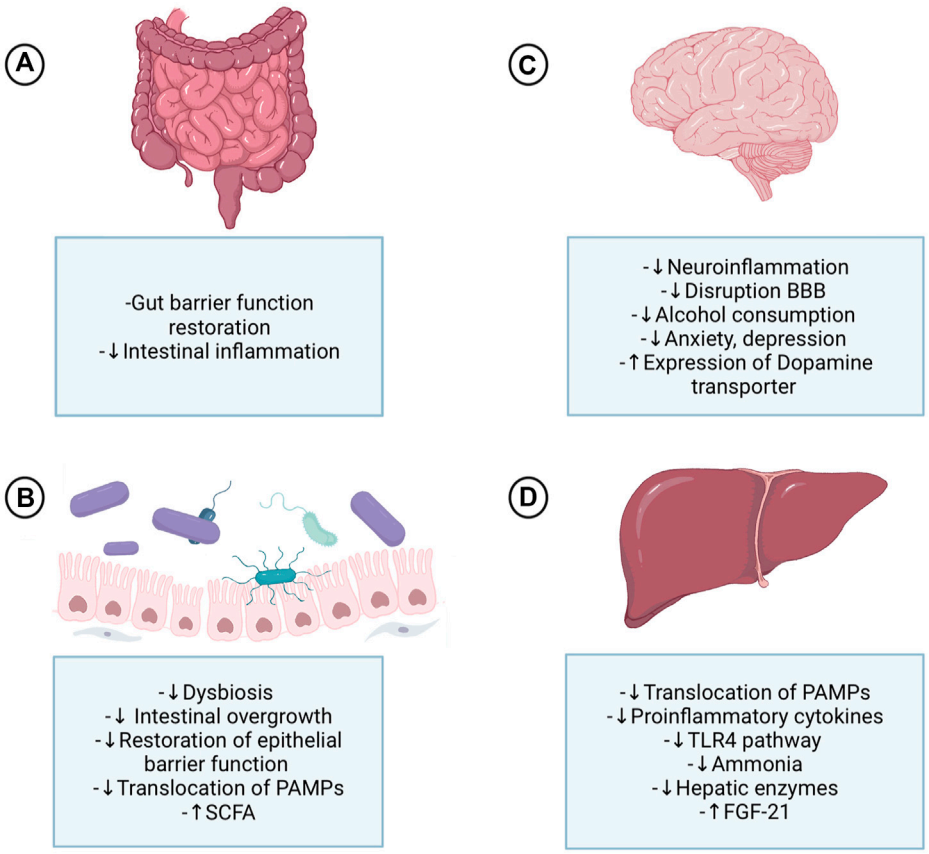

FIGURE 2 | Probiotics' effect on the gut-microbiota-liver-brain axis in ALD. Probiotics exert their actions at different levels of the gut-microbiota-liver-brain axis, acting directly on each of these organs and indirectly due to the axis component's interplay. **(A)** At the intestinal level, probiotics improve digestion and tight junction's expression and are a protective factor for the crypts and mucous layer. **(B)** The change enhances the effects that probiotics produce in the microbiota, restoring it and decreasing dysbiosis triggered by alcohol abuse, which will lead to a decrease in harmful bacteria and an increase in beneficial ones, therefore reducing the high permeability of the gut and the translocation of PAMPs to the liver. **(C)** Probiotics' effect in the brain causes a decrease in proinflammatory cytokines at the systemic level; consequently, the system and neuroinflammation are attenuated by a probiotic-based therapy. Inflammation control is one of the mechanisms behind controlling alcohol consumption and psychological symptoms, such as anxiety and depression. Furthermore, the control of high permeability and the translocation of substances contributes to controlling the disruption of the blood-brain barrier and neuroinflammation. Finally, FGF21 has an important effect on the brain since it produces dopamine transporter transcription in the nucleus accumbens, allowing less dopamine to access the postsynaptic receptor. **(D)** Probiotics have demonstrated multiple benefits at the liver level since the decrease of steatosis to encephalopathy and cirrhosis. These liver effects are explained by the decrease of PAMPs in the systemic circulation, especially LPS, that induce the normalization of the inflammatory processes that are associated, among others, with the TLR4 pathway. Consequently, the adverse effects of alcohol on the liver are decreased; less activation of Küpffer cells, decreased liver enzymes, proinflammatory cytokines, and less fibrosis. Some probiotics cause increased formation of FGF21 in the liver, which has effects on the brain. ALD: Alcoholic liver disease; SCFA: Short-chain fatty acids; PAMPs: Pathogen-associated molecular patterns; BBB: Blood-brain barrier; TLR4: Toll-like receptor 4; FGF21: fibroblast activation protein 21.

damage, probably because this group is associated with a high intestinal permeability that causes bacterial translocation. Other probiotics approaches have also been shown to stimulate intestinal epithelial cell growth, improving the barrier function (Yan et al., 2007).

As noted above, the potential use of probiotics in ALD has already been demonstrated. Evidence has also shown the capacity of probiotic *Lactobacillus reuteri* to produce antimicrobial peptides that prevent the growth of pathogenic bacteria in the intestine (Jones and Versalovic, 2009). *Lactobacillus rhamnosus* GG (LGG) was also shown to reduce alcohol-induced intestinal translocation, oxidative stress, and inflammation in the liver and intestine in a rat model of alcoholic steatohepatitis (Forsyth et al., 2009); all these alterations are involved in ALD. LGGs can also increase intestinal fatty acids and amino-acid metabolism (Shi et al., 2015; Li et al., 2016). Furthermore, studies in rats using LGG conclude that it can reverse established alcoholic hepatic steatosis and injury (Li et al., 2016). Probiotics' direct or indirect improvement of liver function can also be demonstrated based on its effect on restoring ALT levels and AST, lactate dehydrogenase, and total bilirubin described as liver damage biomarkers (Zhang et al., 2019).

Immune response can be also modulated by probiotics. In this matter, a restoration of neutrophil phagocytic capacity has been observed in patients with alcoholic cirrhosis treated with a probiotic scheme based on *Lactobacillus casei* Shirota. Indeed, together with an increase of this activity in this immune cell type, a normalization of the TLR4 receptor expression was also

TABLE 1 | Probiotics based treatment in ALD.

Intervention (probiotic treatment)	Species	Design and model	Summary of results	Mechanism
Lactobacillus Rhamnosus GG 10^{10} Colony-forming unit (CFU)/mL for 1 month Nanji et al. (1994)	Rat	A liquid diet containing ethanol and corn oil for 1 month was administered, followed by administration of *Lactobacillus Rhamnosus*	Improved alcoholic liver disease pathology score and lowered plasma endotoxin level Improved liver enzymes Reduced hepatic steatosis and injury	Reduce plasma endotoxin level, improved barrier, and immune function
Lactobacillus plantarum (TSP05), *Lactobacillus fermentum* (TSF331), and *Lactobacillus reuteri* (TSR332) Hsieh et al. (2021)	Mice	Group A, an ethanol-containing diet (28% ethanol); group B, an ethanol-containing diet + strain TSP05 8.2×10^9 CFU/kg; iv) group C, an ethanol-containing diet + strains TSF331 and TSR332 8.2×10^9 CFU/kg; v) group D, an ethanol-containing diet + strains TSP05, TSF331 and TSR332 8.2×10^9 CFU/kg; and vi) group E, fed a regular diet + strains TSP05, TSF331 and TSR332	Neutralized free radicals and displayed high antioxidant activity *in vitro*	Reduce oxidative stress and inflammatory responses, thus preventing ASH development and liver injury
Bifidobacterium bifidum 0.9×10^8 CFU and *Lactobacillus plantarum* 8PA3 0.9×10^9 CFU Kirpich et al. (2008)	Human	Alcoholic adults were treated with probiotic therapy	Restoration of bowel flora significantly reduces ALT, AST, GGT, LDH, and total bilirubin	Restoring normal bacteria levels improves intestinal barrier function
Bifidobacterium breve ATCC15700: 200 µL of ATCC15700 suspension at the final dose of 10^{10} cells Tian et al. (2020)	Mice	Ethanol-treated mice (alcoholic group) were given alcohol (3.8 g/kg body weight, 200 µL) 1 hour after probiotic administration	Significant reduction of inflammatory cytokines (including TNF-α, IL-1β, IL-6, and IL-17) in both serum and liver	ATCC protects alcohol-exposed mice against liver injury
Bifidobacterium, *Lactobacillus*, and *Streptococcus* (VSL#3) Gupta et al. (2021)	Patients with Chronic liver diseases, including alcoholic cirrhosis and cirrhosis with HE	Patients with these conditions were treated with probiotics	Improved malondialdehyde Improved proinflammatory cytokines (TNF-α, IL-6, and IL-10) in alcoholic cirrhosis patients Improved AST, ALT, GGT in alcoholic cirrhosis patients	Protect against alcohol-induced intestinal barrier dysfunction
Lactobacillus Acidophilus Ziada et al. (2013)	Humans	Ninety patients with ALD were divided into three groups. Group A was treated with lactulose, group B with *Lactobacillus* acidophilus, and group C was a control	Improved neuro metabolites and psychometric analysis Decreased glutamine and glutamate/creatinine ratio	Improved ammonia in the blood
Faecalibacterium prausnitzii, *Bifidobacterium*, and others Kirpich et al. (2008)	Humans	Randomly, patients received 5 days of *Bifidobacterium bifidum* and *Lactobacillus plantarum* 8PA3 or standard therapy (withdrawal + vitamins)	Improved intestinal barrier integrity and ameliorated alcohol-induced liver damage	Gut microbiota alteration by changing secretion of specific metabolites involved in gut barrier dysfunction
Lactobacillus casei Shirota Stadlbauer et al. (2008).	Humans	4-weeks administration of *Lactobacillus casei Shirota* to alcoholic patients	Patients with cirrhosis improved the phagocytic capacity of neutrophils	Probiotics reduce the endotoxemia generated by LPS, increasing neutrophil's function via IL-10 normalization
Lactobacillus Rhamnosus GG 5×10^9 CFU Ezquer et al. (2021)	Rats	Rats were allowed concurrent two-bottle choice access to 10 and 20% (v/v) ethanol solution and water	Pronounced increase in plasmatic FGF21 levels	Activation of dopamine transporter transcription in nucleus accumbens, thus allowing less dopamine to access the postsynaptic receptor

observed in treated patients, suggesting a decrease in inflammatory signals induced by pathogenic ligands (Stadlbauer et al., 2008). Based on the above, by reducing systemic inflammation, we can expect a positive impact of the probiotic on the CNS that could be useful to control the desire to consume alcohol. It was demonstrated that some probiotics reduce the systemic TNF-α and IL-10 levels as well. The study concluded that in mice injected with LPS and D-galactosamine, pretreatment with the probiotic mixture VSL#3 prevented colonic barrier function breakdown, reduced bacterial translocation, reduced TNF-α levels tissues, and significantly attenuated liver injury (Ewaschuk et al., 2007). Studies have shown that the use of *Lactobacillus spp,* including *Lactobacillus plantarum* and Fructo-oligosaccharides, reduces the production

of primed TNF-α by peripheral blood mononuclear cells in cirrhotic patients (Riordan et al., 2003). On the other hand, *in vitro* studies demonstrated that *Bifidobacteria* induce the production of IL-10 by cultured human dendritic cells, capable of modulating the immune system (Hart et al., 2004). Other studies of the effect of *Bifidobacteria longum* and *Lactobacillus acidophilus* in inhibiting plasma lipid peroxidation showed that both intestinal strains could protect plasma lipids from oxidation to different degrees (Lin and Chang, 2000). Additionally, some probiotics regulate the host defense peptides response by inducing the expression of antimicrobial peptides (AMPs). In fact, the probiotic *Escherichia coli* strain Nissle (EcN) and some species of Lactobacilli induced a high expression of human beta-defensin-2 in epithelial cells. Similarly, other probiotics, such as *Lactobacillus reuteri,* can increase the secretion of interleukin-22 (IL-22), which mediate intestinal mucosa repair and defense *via* AMPs induction (Wehkamp et al., 2004; Gaudino et al., 2021; Patnaude et al., 2021).

In line with this cumulative evidence, there is a particular interest in supporting the use of probiotics in ALD treatment. Targeting the microbiota-gut-liver axis with this approach allows introducing a holistic therapy to manage the multifactorial pathogenesis of ALD.

Probiotics Benefit Addiction and Neuroinflammation

Alcohol dependence is considered an epiphenomenon of systemic neuroinflammation. Although the mechanisms underlying this relationship are not fully described, it has been shown that alcohol and derived metabolites can modify some brain neurotransmitter signals, including γ-aminobutyric acid (GABA), glutamate, and dopamine circuits, with this effect influenced by the inflammation induced by changes of intestinal microbiota. Studies based on the use of magnetic resonance spectroscopy have demonstrated a high glutamine/glutamate to creatinine ratio in alcoholic patients with hepatic encephalopathy (Gupta et al., 2021). Interestingly, it has been observed that *Lactobacillus* and *Bifidobacterium* can metabolize glutamate, an excitatory neurotransmitter that regulates glutamine/glutamate signaling, to produce GABA in the gut. As an inhibitory neurotransmitter, GABA acts locally, regulating the information relayed from the gut to the brain. Remarkable findings from a recent clinical study published by Morley K. et al. revealed an inverse correlation between GABA levels in the brain and ALD severity (Morley et al., 2020), suggesting that *Lactobacillus* and *Bifidobacterium* could be an interesting therapeutical approach to modulate this neurotransmission pathway in this pathology (Gupta et al., 2021). Indeed, a long-term diet supplemented with multi-species live *Lactobacillus* and *Bifidobacterium* mixture has been demonstrated to enhance cognitive and memory functions by altering GABA concentrations in the brain in a middle-aged rat model (O'Hagan et al., 2017).

In line with this evidence, it has been demonstrated that administering the probiotic *Lactobacillus rhamnosus* increases plasma levels of fibroblast growth factor 21 (FGF21), a transcriptional activator of the dopamine transporter in dopaminergic neurons at the nucleus accumbens of Wistar-derived high drinker UChB rats (Ezquer et al., 2021). Considering the role of dopamine in addiction, increased reuptake of this neurotransmitter in the synaptic cleft due to increased transporter activity induced by this probiotic suggests that this mechanism is responsible for reward reduction alcohol intake in this model. Based on this evidence, it is easy to imagine that a probiotics-based complementary therapy to ALD treatment might diminish disease progression mediated by reducing lower alcohol consumption.

In recent years, probiotics' impact on the expression of brain receptors involved in addiction, such as dopamine receptor 1 (DR1) and DR2, has been studied. It has been observed that alcohol and other substances can increase dopamine release, generating a sensation of pleasure and leading the subject to repeat a specific behavior. Alcohol acts directly on GABA receptors, positively modulating dopamine release in the nucleus accumbens and the ventral tegmental area (Grace et al., 2007; Koob and Volkow, 2010). According to the aforementioned study conducted by Jadhav KS. et al., the vulnerable group of rats showed a loss of control over alcohol intake associated with a significantly high DR1 expression and lowered DR2 expression in the striatum compared to the resilient group. The study correlated these alterations with intestinal microbiota changes observed in vulnerable rats, suggesting that gut microbiota composition may contribute to inhibitory innervations in addiction-related brain circuits. Although the correlation observed requires further investigation, particularly to discover the mechanism that explains how gut microbiota induces striatal dopamine receptor expression, a positive correlation between D2R mRNA expression and a low abundance of bacteria of the Firmicutes phylum was observed. This phylum includes bacteria of the Clostridial order, which together with the *Ruminococcacea* and Lachnospiraceae, were positively associated with AUD severity. Thus, DR2 could be an interesting target to achieve by probiotics-based therapeutic approaches to restore intestinal Lachnospiraceae and *Ruminococcacea* levels (Jadhav et al., 2018).

Additional proposals aimed at intestinal microbiota modulation have also been explored in AUD. It was shown that fecal microbiota transplantation from a healthy donor with high levels of Lachnospiraceae and Ruminococcaceae drove a short-term reduction in craving and consumption of alcohol in patients with alcoholic cirrhosis associated with intestinal microbial changes. Microbial diversity increased with higher Ruminococcaceae and other SCFAs producing taxa, linked with SCFA levels following fecal microbiota transplantation but not placebo (Bajaj et al., 2021). Interestingly, a trend toward higher SCFA levels in stool and plasma was detected in a post-FMT group, positively associated with Lachnospiraceae and Ruminococcaceae constituent. The intermediary role of SCFA in the communication of the gut-brain axis in addiction disorders, in both animal and human models, has been well described. Therefore, increased SCFA content post-FMT suggests this factor as a potential mediator of alcohol addiction behavior (Bajaj et al., 2021).

Based on the above, probiotics-based treatment may be an interesting intervention to reduce alcohol intake and disease progression by restoring gut microbiota and improving microbiota-gut-liver-brain axis communication.

DISCUSSION

Considering that alcohol addiction is a biopsychosocial condition, an integrative treatment is required to achieve better clinical response, greater adherence, and reduced costs associated with the disease, in both the short and long term. In this matter, novel therapeutical approaches have emerged from research efforts toward discovering possible therapeutic targets. Among them, probiotic discovery and development, and gene editing therapy of enzymes, such as alcohol dehydrogenase and aldehyde dehydrogenase, to complement pharmacological and psychological interventions are currently being used.

Along with probiotics, prebiotics has also emerged as a complementary therapy. Both have been recently included in a category denominated "psychobiotics" characterized by their potential benefits for the CNS (Ansari et al., 2020). Prebiotics was described in 1995 as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health" (Gibson and Roberfroid, 1995). This terminology has remained so far, and it has been observed that they serve as an energy source for microbiota, regulating its composition, functions, and the intestinal environment (Davani-Davari et al., 2019). Furthermore, various studies using a combination of probiotics and prebiotics (symbiotics) in an animal model have been assays, and some formulas, based on specific mixed, have been explored in clinical practice (Markowiak and Śliżewska, 2018). Indeed, increased interest in therapeutical approaches toward microbiota restoration has emerged from diverse studies utilizing prebiotics and probiotics for various conditions such as ALD, addiction, depression, anxiety, autism, schizophrenia, and Alzheimer's. The beneficial outcomes obtained from these interventions, principally from probiotics as the most used, reinforce research effort in this matter.

Regarding probiotic usage safety, it is considered that they lack factors that allow them to develop pathogenic capacities, and the adverse effects related to them are minimal and occur in specific contexts. There are cases of sepsis due to probiotics, mainly Lactobacilli and Bifidobacteria. However, its incidence according to studies is only 0.02%. In any case, it is recommended to exercise greater caution and vigilance in administering probiotics to patients at risk. On the other hand, its use in patients is still considered beneficial due to reducing bacterial translocation. Many studies show positive effects of probiotics, even in the extreme stages of life, and complications related to their use are extremely rare, despite their unrestricted use (Brunser, 2017 #3).

ALD has complex and multifactorial pathogenesis, including environmental factors, genetic predisposition, immune response, and gut microbiota in its development. In this context, its treatment should target many mechanisms involved in its development and its maintenance. To date, abstinence-based therapy remains the best choice for treatment in ALD. However, the increased relapse rate challenges discovering new therapies to achieve integral management of ALD patients. Therefore, diverse therapeutic interventions focused on each component involved in the pathophysiology of ALD have been explored. The study of gut microbiota and its alteration has gained importance recently due to its multiple impacts on individual health, including psychological and behavioral fields. These findings have positioned the microbiota modulator approaches, such as probiotics, prebiotics, fecal transplantation and antibiotics, as a feasible therapeutic option. In this context, using probiotics stands out due to their effective microbiota modulation properties, being accessible and safe compared to other approaches. Probiotics have proved to have many benefits at the microbiota-gut-liver-brains axis level in ALD. They reduce dysbiosis, restore normal microbiota and intestinal permeability, and decrease bacterial products translocation and liver, brain, and systemic inflammation. Even more important is their role in decreasing the progression of the disease by its properties of modulating alcohol addiction at CNS. In addition, some probiotics, like *Lactobacillus rhamnosus*, can be used to decrease alcohol intake due to its proven anti-inflammatory properties, which can prevent ALD progression and establishment.

This review provides updated evidence of the role of probiotics not only in the pathogenesis of ALD but also in reducing craving and alcohol consumption. Future research is necessary to support the use of probiotics to decrease the severity and progression of ALD, as well as to evaluate its impact on the interaction in the microbiota-gut-liver-brain axis in other addictive disorders.

AUTHOR CONTRIBUTIONS

CF and MSD: drafting the original manuscript. CF, MSD, LV-P, and CJB conceived the idea and the aim of the review. CJB, LV-P, JP, and JR: reviewing. CJB and LV-P: editing. CF, MSD, JP, JR, LV-P, and CJB: validation. LV-P and CJB: supervision. LV-P and CJB: funding acquisition. All authors contributed to the article and approved the submitted version.

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Bifidobacterium Strains Present Distinct Effects on the Control of Alveolar Bone Loss in a Periodontitis Experimental Model

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Periodontitis is an inflammatory disease induced by a dysbiotic oral microbiome. Probiotics of the genus *Bifidobacterium* may restore the symbiotic microbiome and modulate the immune response, leading to periodontitis control. We evaluated the effect of two strains of *Bifidobacterium* able to inhibit *Porphyromonas gingivalis* interaction with host cells and biofilm formation, but with distinct immunomodulatory properties, in a mice periodontitis model. Experimental periodontitis (P+) was induced in C57Bl/6 mice by a microbial consortium of human oral organisms. *B. bifidum* 162^{2A} [B+ (1622)] and *B. breve* 110^{1A} [B+ (1101)] were orally inoculated for 45 days. Alveolar bone loss and inflammatory response in gingival tissues were determined. The microbial consortium induced alveolar bone loss in positive control (P + B-), as demonstrated by microtomography analysis, although *P. gingivalis* was undetected in oral biofilms at the end of the experimental period. TNF-α and IL-10 serum levels, and Treg and Th17 populations in gingiva of SHAM and P + B- groups did not differ. *B. bifidum* 162^{2A}, but not *B. breve* 110^{1A}, controlled bone destruction in P+ mice. *B. breve* 110^{1A} upregulated transcription of *Il-1β*, *Tnf-α*, *Tlr2*, *Tlr4*, and *Nlrp3* in P-B+(1101), which was attenuated by the microbial consortium [P + B+(1101)]. All treatments downregulated transcription of *Il-17*, although treatment with *B. breve* 110^{1A} did not yield such low levels of transcripts as seen for the other groups. *B. breve* 110^{1A} increased Th17 population in gingival tissues [P-B+ (1101) and P + B+ (1101)] compared to SHAM and P + B-. Administration of both bifidobacteria resulted in serum IL-10 decreased levels. Our data indicated that the beneficial effect of *Bifidobacterium* is not a common trait of this genus, since *B. breve* 110^{1A} induced an inflammatory profile in gingival tissues and did not prevent alveolar bone loss. However, the properties of *B. bifidum* 162^{2A} suggest its potential to control periodontitis.

Keywords: periodontitis, *P. gingivalis*, probiotics, immune modulation, *Bifidobacterium*

INTRODUCTION

Periodontitis comprises a destructive inflammatory process of the teeth supporting tissues induced by a dysbiotic subgingival microbiome (Hajishengallis, 2014). *Porphyromonas gingivalis* is considered a key stone pathogen in periodontitis, allowing the appropriate conditions to induce the disease (Kuboniwa et al., 2017), due to the ability to alter the subgingival ecosystem caused by its strategies to evade the immune system (Chopra et al., 2020). The dysbiotic microbiome of periodontitis involves not only increased levels of *P. gingivalis* (Hajishengallis, 2014), and other pathogens, but also pathobionts as *Streptococcus gordonii* (Kuboniwa and Lamont, 2010; Socransky et al., 1998), *Prevotella intermedia* (Barbosa et al., 2015), and *Fusobacterium nucleatum* (Polak et al., 2017) and decreased levels of beneficial bacteria (Hajishengallis, 2014). The mechanical periodontal treatment and systemic antimicrobials are able to reduce periodontal pathogens in subgingival regions (Teughels et al., 2020). However, their effect is not entirely predictable, and long-term success requires the establishment of a program of supportive periodontal therapy following the treatment of active disease (Armitage and Xenoudi, 2016).

The ecological shift of the periodontal microbial community towards disease may be hindered by the oral administration of living beneficial bacteria with antimicrobial and immunomodulatory properties, considered as probiotics. Hence, probiotics may comprise an argued ecological therapeutic approach to control periodontitis (Matsubara et al., 2016). Probiotics may directly interfere with pathogen's colonization by competition for adhesion sites in oral surfaces and/or in already adherent bacteria in the biofilm and production of antimicrobial substances, or indirectly, by modulating host immune response and by decreasing permeability of the epithelial barrier of mucosa surfaces (Teughels et al., 2011).

Periodontal tissues destruction is induced by an exacerbated response triggered by the dysbiotic microbiome after recognition of pathogen-associated molecular patterns (PAMPS) and damage-associated molecular pattern molecules (DAMPs) by extracellular and intracellular pattern recognition receptors (PPRs) such as Toll-Like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-Like receptors (NLRs). Recognition by PPRs activates transcription factors, inducing the production of inflammatory cytokines and chemokines (Ojcius and Saïd-Sadier, 2012).

Periodontitis is characterized by an elevated pro-inflammatory: anti-inflammatory ratio, with increased levels of cytokines such as IL-1β, IL-6 and TNF-α (Kawamoto et al., 2020). IL-1β is a major pro-inflammatory cytokine in periodontitis (Aral et al., 2020), and its levels in gingival tissue are related to disease severity (Hou et al., 2003). IL-1β inactive precursor pro-IL-1β is only converted to its biologically active form after inflammasome activation. Inflammasomes are multiprotein complexes constituted by an intracellular receptor, an adaptor protein ASC (apoptosis-associated speck-like protein containing CARD - caspase-recruitment domain) and a pro-caspase 1 (Shibata, 2018). Receptor recognition leads to inflammasome activation, in that ASC converts pro-caspase-1 to caspase-1, which cleaves pro- IL-1β, pro-IL-18, and pro-IL-33 to their active forms, and/or induce cell death by pyroptosis (Aral et al., 2020). There are several types of inflammasome differing on their receptor molecules, activation molecules and tissue's locations (Man et al., 2016; Abderrazak et al., 2015). NLRP3 (Nod-like receptor pyrin domain-containing protein 3) and AIM-2 (Absent in melanoma 2) inflammasomes are associated to periodontal disease, and high levels of their receptors, NRLP3 and AIM-2, are detected in gingival tissues of periodontitis patients (Aral et al., 2021).

The immune response to microbial insults in the gingival tissues also involves T cells proliferation, differentiation towards Th17 subsets, and induction of regulatory T cells (Treg) (Silva et al., 2015). Pathogens induce T cells polarization to Th17 in gingival tissues (Moutsopoulos et al., 2012), whereas inhibition of Treg increases periodontal inflammation and bone resorption (Garlet, 2010). Despite their role in homeostasis, Tregs can also differentiate into the Th17 effector subtype under inflammatory conditions, in order to mount a defense against extracellular pathogens (Round and Mazmanian, 2010).

Bifidobacterium are probiotics commonly used in humans (Gupta, 2011), usually considered safe (Di Gioia et al., 2014) and showed encouraging results in controlling ligature-induced periodontitis in rats by modulating the host response (Oliveira et al., 2017). In humans, the intake of *B. animalis* subsp. *lactis* led to reduced plaque and gingival indexes and decreased IL-1β levels in gingival crevicular fluid (Toiviainen et al., 2015) and was successfully used as an adjunct to the mechanical treatment of periodontitis (Invernici et al., 2018).

Certain mechanisms of probiotics are common to *Bifidobacterium* spp, such as their antimicrobial properties through the production of lactic acid (Gillor et al., 2008), which impacts *P. gingivalis* survival (Jäsberg et al., 2016). We have also shown that bifidobacteria such as *B. bifidum* 162^{2A} *and B. breve* 110^{1A} may also impair *P. gingivalis* colonization in an acid-independent mechanism, by favoring commensals over the pathogen, altering the transcription of virulence encoding genes (Ishikawa et al., 2020) and by reducing its adhesion and invasion to gingival epithelial cells (GECs) (Albuquerque-Souza et al., 2019). However, beneficial properties of probiotics are strain specific, and the most appropriate probiotic strains and their mechanisms in controlling periodontal destruction were still not determined (Mulhall et al., 2020). We previously showed that the *in vitro* overall effect of *B. bifidum* 162^{2A} on *P. gingivalis* seemed more pronounced than of *B. breve* 110^{1A}, including its impact on transcription of virulence factors (Ishikawa et al., 2020). Furthermore, *B. bifidum* 162^{2A} and *B. breve* 110^{1A} also showed different effects on the production of IL-1β by *P. gingivalis* infected GECs (Albuquerque-Souza et al., 2019).

The *in vitro* determination of potential candidates is the first step to select a probiotic strain, but *in vivo* experimental data are needed to elucidate its benefits to health and underlying mechanisms. Thereby, we aimed to evaluate the effect of *B. breve* 110^{1A} and *B. bifidum* 162^{2A}, with known activities over *P. gingivalis* but distinct immunomodulatory properties, in a periodontitis experimental model.

MATERIALS AND METHODS

Animals and Group Allocation

Ninety-six, 4 weeks old C57Bl/6 male mice, bred under Specific Pathogen Free conditions were acquired from the Central Facility of School of Medicine, University of São Paulo, and maintained in the mouse breeding facility of the Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, in collective microisolators containing up to four animals, with an artificial light-dark cycle of 12 h, at a constant temperature of 22°C, and water and food available ad libidum. Animals were randomly allocated in six groups (n = 8), and two independent assays were performed. Experimental groups received a microbial consortium (P+) and/or *B. breve* 110^{1A} [B+ (1101)] or *B. bifidum* 162^{2A} [B+ (1622)]. Controls were inoculated with vehicles of microbial consortium (P-) and/or probiotics (B-). The groups were as follows: SHAM (P-B-) (negative control); Positive control (P + B-) (microbial consortium and probiotic vehicle); Bifidobacteria control groups P-B+ (1101) and P-B+ 1622) (microbial consortium vehicle and *B. breve* 110^{1A} or *B. bifidum* 162^{2A}); and experimental groups [P + B+ (1101) and P + B+ (1622)]. The animals were monitored for weight gain, loss of mobility, and skin appearance throughout the experimental period. All procedures were performed following National Institutes of Health guidelines for experimental animal welfare and approved by the Institutional Animal Care and Use Committee (ICB USP Approval number:1112017).

Blinding

Each animal was assigned a temporary random number within the group. Based on their position on the rack, cages were given a numerical designation. For each group, a cage was selected randomly from the pool of all cages. Blinding was carried out during the allocation, evaluation of the results, and data analysis. Blindness was unfeasible during the experiment since the same researcher prepared and inoculated the organisms. Furthermore, the bacterial suspensions differed in color from the vehicle.

Exclusion Criteria

Animals presenting alteration in growth, weight and/or physical defects at baseline were excluded.

Sample Size

Sample calculation was performed using alveolar bone loss as the primary outcome, based on data obtained in a pilot study. Taking into consideration a difference in the bone volume of 4,719 cubic pixels at a standard area, a sample size of 7.84 animals was adequate to obtain a Type I error rate of 5% and power greater than 80% (Charan and Kantharia, 2013). Thus, each experimental group was formed by eight animals.

Orally Administered Cultures of Microbial Consortium and Bifidobacteria

Bifidobacterium breve 110^{1A} and *Bifidobacterium bifidum* 162^{2A} isolated from fecal samples of healthy children in Bahia and part of the biobank of the Federal University of Minas Gerais were tested (approval by the Ethics Committee of the Federal University of Bahia 276/2009) (Souza et al., 2013). The microbial consortium for inducing experimental periodontitis comprised: *P. gingivalis* ATCC 33277 (non-capsulated, fimbriated, genotype fimA I), *P. gingivalis* W83 (capsulated K1, afimbriated, genotype fimA IV), *Prevotella intermedia* 17 (Fukushima et al., 1992), *Fusobacterium nucleatum* ATCC 25586 (Barker et al., 1982) and *Streptococcus gordonii* DL1 (Pakula, 1965).

Bacteria from frozen stocks were cultivated in agar plates, transferred to broth, and grown to reach the stationary phase. *P. gingivalis*, *P. intermedia* and *F. nucleatum* were grown in BHI HM broth (Brain Heart Infusion Broth supplemented with 1 mg Hemin/mL and 0.1 mg Menadione/mL), in an anaerobic chamber (PlasLabs, Lansing, MI, United States) containing an atmosphere of 85% N_2, 5%H_2 and 10% CO_2. *S. gordonii* was cultivated in BHI broth under microaerophilic atmosphere (10% CO_2). *Bifidobacterium* strains were grown in MRS broth under anaerobiosis. Standard cultures were obtained for each strain, cells were harvested and resuspended in 500 µL lyophilization solution [10% skin milk with 5% L-Glutamic acid monosodium salt hydrate, and 5% dithiothreitol (Sigma-Aldrich Darmstadt, Germany)]. Aliquots were lyophilized using Freezone Triad Freezer Dryers (Freezone Triad Freezer Dryers, Labconco, Kansas City, MI, United States) at −40°C, under vacuum and maintained at −80°C. Viability was estimated for each lot under appropriate conditions.

Experimental Treatments

Before the beginning of the experimental period, the mice resident microbiota was reduced by adding 1 mg kanamycin/mL (Gatej et al., 2018) and 1 mg amoxicillin/mL to the drinking water for 4 consecutive days, and the oral cavities were rinsed with 2% chlorhexidine digluconate (Peridex; Procter and Gamble) using a microbrush (Kang et al., 2012) for 2 days, followed by a 2-days washout period. At the first day of the experimental period, lyophilized *B. breve* 110^{1A} and *B. bifidum* 162^{2A} were suspended at 2×10^{10} CFU/mL in PBS/2% carboximethylcellulose gel (Gatej et al., 2018) and 50 µL aliquots were administered in the oral cavity with a gavage needle to groups [B+(1101)] or [B+(1622)]. This procedure was repeated daily for 45 days.

Lyophilized bacteria of the microbial consortium were inoculated in BHI HM broth, incubated for 6 h under anaerobiosis to recover to physiological state and suspended in PBS/2% carboximethylcellulose gel (Kang et al., 2012; Gatej et al., 2018) to reach 2×10^{12} CFU/mL of each strain. Viability of each strain was confirmed. 50 µL aliquots (containing 1×10^{11} CFU/each strain) were inoculated in the oral cavity of P+ groups 5 days/week for 5 weeks, totalizing 25 inoculations of the microbial consortium, starting at day 3 of the experimental period and ending 12 days before euthanasia. In order to avoid a direct effect of the bifidobacteria to the viable bacteria of the microbial consortium, the bifidobacteria were orally inoculated in the morning, whereas the microbial consortium was given 6 h later.

Control groups (P- and/or B-) received only vehicle (PBS/2% carboximethylcellulose gel) at the same regimen used for the infected groups (P+ and/or B+).

Samples Collection

Forty-five days after the initial inoculation with bifidobacteria, the mice were anesthetized with ketamine/xylazine, blood was obtained by intracardiac puncture, and serum stored at −80°C. The mice were euthanized by cervical dislocation. Oral biofilm samples were obtained with sterile microbrushes and placed in Tris EDTA (TE) buffer (pH 8.0). Gingival tissue was collected from the maxilla around molars (Mizraji et al., 2013), and half transferred to RNAlater™ Stabilization Solution (Invitrogen by Thermo Fisher Scientific, Vilnius, Lithuania) for gene expression analysis and half to RPMI 1640 cell culture medium (Sigma–Aldrich, St. Louis, MO, United States) for immune cells evaluation. Then, a hemimaxilla was transferred to 4% formaldehyde solution for 24 h, transferred to PBS and stored at 4°C for alveolar bone analysis.

Alveolar Bone Analysis

Alveolar bone resorption was determined by microtomography (MicroCT) using a microtomograph (SkyScan 1176 version 1.1, Kontich, Belgium) at 45 kV voltage, 550 uA current, 8.71 μm pixel size, 0.2 mm aluminum filter. The left hemimaxillae were scanned, and a blinded examiner selected a standard area of 60 × 30 pixel at the interproximal region between first and second M in 15 coronal sections from the second M ECJ. The images were analyzed by calculating bone volume, percentage of bone volume, and total porosity using CTAnalyser software Version 1.15.4.0, Skyscan (Rogers et al., 2007).

Gene Expression Analysis

RNA was extracted from gingival samples using TRizol LS Reagent (Invitrogen Life Technologies, Carlsbad, CA, United States) and Mini-BeadBeater (BioSpec 3110BX Mini-BeadBeater-1 High Energy Cell Disrupter, Campinas, São Paulo, Brazil) for 20 s, twice. The resulting RNA was treated with desoxyribonuclease (Ambion™ DNase I, Invitrogen Life Technologies, Carlsbad, CA, United States). cDNA was obtained using the SuperScript™ Vilo™ Synthesis Kit for RT-PCR (Invitrogen Life Technologies). Quantitative PCR was performed in StepOne Plus System thermocycler (Applied Biosystems, Foster City, CA, United States). Each reaction was performed with 100 ng cDNA using TaqMan™ Gene Expression Assay (Invitrogen by Thermo Fisher Scientific, Vilnius, Lithuania). Commercial Taqman primers and probes (Invitrogen Life Technologies, Carlsbad, CA, United States) comprised Tlr-2 (Mm01213946_g1), Tlr-4 (Mm00445273_m1), Nlrp3 (Mm04210224_m1), Il-1β (Mm00434228_m1), Il-17 (Mm00439619_m1), Tnf-α Mm00607939_s1) and β-actin (Mm00607939_s1). Relative expression of target genes was calculated by the ΔΔCT method, using β-actin as endogenous control (Pfaffl, 2001), and expressed as fold changes in relation to control group (SHAM).

P. gingivalis Detection in Oral Biofilm

DNA was extracted using Meta-G-Nome™ DNA Isolation kit - MGN0910 (Epicentre, Madison, WI, United States). *P. gingivalis* was detected by real-time PCR, using species-specific primer pairs (5′-TGTAGATGACTGATGGTGAAAACC-3′ and 5′-ACGTCA CCACCTCCTTC-3′) (Amano et al., 2000). The reaction consisted of 10 ng DNA, 25 pMol of each primer, and SYBR® Green Real-Time PCR Master Mix (Invitrogen Life Technologies, Carlsbad, CA, United States) and was performed at 50°C/2 min, 95°C/10 min, followed by 40 cycles at 95°C/15 s, 60°C/1 min, in a StepOne Plus thermocycler (Applied Biosystems, Foster City, CA, United States). The standard curve consisted of serially diluted *16SrRNA* of *P. gingivalis* ATCC 33277 amplicon. Efficiency was estimated as 100 ± 10%, and the data were reported as number of *P. gingivalis* 16SrRNA copies/μg DNA.

Tregs and Th17 Cells Populations in Gingival Tissue

The percentages of $CD45^+CD3^+CD4^+$ T cells, $Foxp3^+$(Treg) or $ROR\gamma t^+$ (Th17) subpopulations in gingival tissue samples were determined by flow cytometer analysis (Souto et al., 2014). Gingival samples were pooled for every four animals due to the low amount of total cells populations, and dissociated with 0.28 Wunsch/mL liverase blendyme (Gibco by Life Technologies, New York, NY, United States), using Tumor Dissociation mouse kit (MiltenyiBiotec Inc., Auburn, Al, United States) with the aid of the MACS™ Octo Dissociator with Heaters (MiltenyiBiotec Inc., Auburn, Al, United States). Death cells and cell debris were distinguished from viable cells by staining with Fixable Viability Stain 570 [BD Horizon™ Fixable Viability Stain 570 (Becton; Dickinson and Company, San Diego, CA, United States)]. Then, 1–10 × 10^6 viable cells were stained using fluorescence-bound Antibodies [APC-Cy™ seven Rat Anti-Mouse CD3, FITIC Rat Anti-Mouse CD4 and BV510 Rat Anti-Mouse CD45 (Becton; Dickinson and Company, San Diego, CA, United States)]. Cells were then permeabilized and fixed [BD Pharmingen™ Mouse Foxp3 Buffer Set kit (Becton; Dickinson and Company, San Diego, CA, United States)], and intracellular staining was performed with Alexa Fluor® 647 Rat anti-MOUSE Foxp3, e BV421 Mouse Anti-Mouse RORγt, overnight, at 4°C. Non-specific binding was blocked by BSA. Unstained samples were used as negative controls, and BD™ CompBeads (Becton; Dickinson and Company, San Diego, CA, United States) labeled used for staining compensation. Data from 100,000 events were acquired using the BD FACSCanto™ II cytometer (Becton; Dickinson and Company, San Diego, CA, United States), and analyzed using FlowJo 10.6 software (Becton; Dickinson and Company, San Diego, CA, United States).

Serum Cytokines Levels

IL-10 and TNF-α levels in serum were evaluated by ELISA, using the BD Opteia Mouse ELISA Set kit (Becton; Dickinson and Company, San Diego, CA, United States). OD was determined at 450 nm in a spectrophotometer [Microplate Manager® Software Version 5.2.1 (Bio-Rad Laboratories, INC., Hercules, CA, United States)]. After comparison to a standard curve, data were expressed in pg/mL.

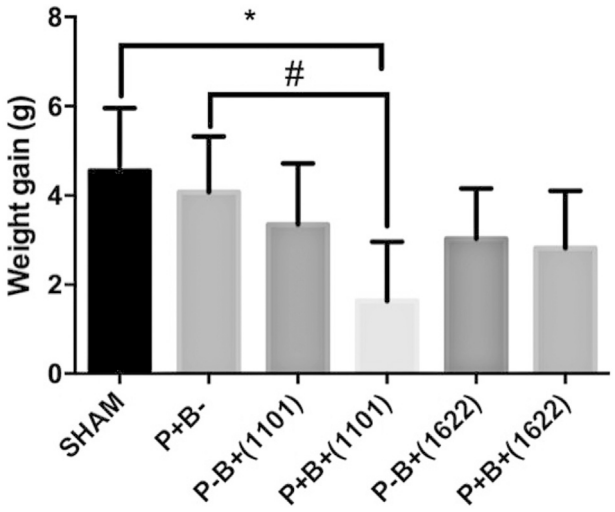

FIGURE 1 | Mean and SD of weight gain in grams after 45 days of experimental period of C57Bl/6 mice submitted to different treatments: SHAM (negative control), P + B- (positive control), P-B+ (1101) (*B. breve* 110^{1A}), P + B+ (1101) (microbial consortium + *B. breve* 110^{1A}), P-B+ (1622) (*B. bifidum* 162^{2A}) and P + B+ (1622) (Microbial consortium + *B. bifidum* 162^{2A}). *Statistically significant difference in relation to negative control (SHAM), # Statistically significant difference in relation to positive control (P + B-). ANOVA, Tukey's multiple comparison, p <0.05%. Data representative of two independent experiments (n = 8 mice/per group).

Statistical Analysis

Data were tested for normality using Kolmogorov-Smirnov test with Lilliefors correlation and homogeneity of variances was assessed by the F test. One-way ANOVA *post hoc* Tukey test was used for determining differences among the studied groups in alveolar bone parameters, relative transcription levels, percentage of Treg and Th17 cells in gingival tissues, and serum cytokines levels. Statistical significance was set at $p < 0.05$. The analyses were performed using the GraphPad Prism® Version 6.0 statistical package (GraphPad software, La Jolla, CA, United States).

RESULTS

The treatments did not result in any observable alteration in skin, hair, or locomotion activity. This *in vivo* study was performed in two independent assays, which gave similar results. Weight gain was similar for all groups, except for the group which received the microbial consortium and *B. breve* 110^{1A} [P + B+ (1101)], which gained less weight than SHAM (**Figure 1**).

Effect of Probiotics on Alveolar Bone Loss

Alveolar bone volume was determined at the interproximal region of first and second M at the left maxilla (**Figure 2A**). Data on bone volume, percentage of bone volume, and bone porosity in the studied groups are shown in **Figures 2B–D**, respectively. The microbial consortium was able to induce significant bone loss, indicated by reduced bone volume and increased porosity in positive control (P + B-) compared to SHAM (P-B-). Alveolar bone volume, percentage of alveolar bone volume, and bone porosity of the groups receiving only bifidobacteria [P-B+ (1101) and P-B+ (1622)] did not differ from SHAM (ANOVA, $p > 0.05$), indicating that *B. bifidum* 162^{2A} and *B. breve* 110^{1A} did not induce alveolar bone loss. Administration of *B. bifidum* 162^{2A} prevented the reduction in bone volume and increase in bone porosity induced by the microbial consortium [P + B+ 1622) ≠ P + B-, $p < 0.05$], whereas administration of *B. breve* 110^{1A} did not.

Gene Expression in the Gingival Tissue

Despite the microbial consortium induced bone destruction, there were no differences in mRNA levels of *Il-1β* and *Tnf-α* or of genes encoding receptors for PAMPs between P + B- and SHAM groups, as shown in **Figures 3A,B**. However, transcription of *Il-17* was down-regulated in P + B- compared to SHAM (**Figure 3C**). The daily administration of *B. breve* 110^{1A} up-regulated transcription of *Il-1β* and *Tnf-α* [P-B+ (1101) and P + B+ (1101) ≠ SHAM], **Figures 3A,B**. All treatments downregulated transcription of *Il-17* when compared to SHAM, but the group treated only with *B. breve* 110^{1A} [P-B+(1101)] showed the highest *Il-17* mRNA levels among the treated groups (**Figure 3C**). The microbial consortium down-regulated transcription of *Il-1β*, *Tnf-α* and *Il-17* in animals receiving *B. breve* 110^{1A} [P + B+(1101) ≠ P-B+(1101)], although *Il-1β* and *Tnf-α* transcript levels in P + B+(1101) were still above those of the P + B- group. Meanwhile, the oral administration of *B. bifidum* 162^{2A} did not interfere in *Il-1β* regulation, down-regulated *Il-17*, but promoted a slight up-regulation (less than 2 folds) in *Tnf-α* transcription [P + B+ 1622) ≠ P + B-], **Figure 3**.

The microbial consortium did not alter the transcription profile of genes encoding receptors to PAMPs in the gingival tissues (**Figures 3D–F**). However, transcription of *Tlr2*, *Tlr4*, and *Nrlp3* was up-regulated by *B. breve*110^{1A} [P-B+ (1101) ≠ P + B- ≠ SHAM]. The positive regulation of *Tlr2* and *Nrlp3* was attenuated by the microbial consortium [P + B+ (1101)]. On the other hand, both *Bifidobacterium* strains induced up-regulation of *Tlr4* in mice that were also challenged with the microbial consortium [P + B+ (1101) and P + B+ 1622) ≠ P + B- ≠ SHAM], **Figure 3E**.

P. gingivalis Levels in Oral Biofilm

P. gingivalis levels were determined by amplification of *16SrRNA* using species-specific primers, and data were normalized by CT values in SHAM. Oral inoculation of microbial consortium did not induce *P. gingivalis* persistent colonization of the oral biofilm, since no group reached CT values above background.

Tregs and Th17 Populations

Immune cells were evaluated by flow cytometry analysis. Gingival tissue samples from four mice were pooled, making two pooled samples/group, due to low amount of tissue. T-helper (Th) cells ($CD4^+$) were stained for $CD45^+CD3^+CD4^+FoxP3^+$ (Tregs cells) and for $CD45^+CD3^+CD4^+ROR\gamma t^+$ (Th17 cells). The percentages of innate immune cells detected are shown in the **Supplementary Table S1**. Percentages of Tregs or Th17 populations were similar

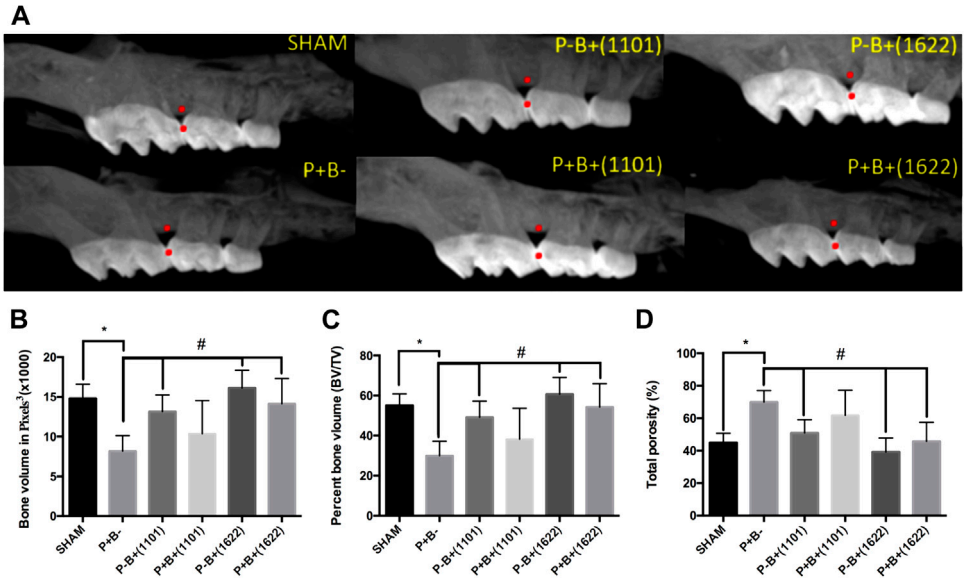

FIGURE 2 | Alveolar bone analysis determined by Microtomography in the interproximal region of first and second M at the right maxilla of C57Bl/6 mice submitted to different treatments for 45 days: SHAM (negative control), P + B- (positive control), P-B+ (1101) (*B. breve* 110^{1A}), P + B+ (1101) (microbial consortium + *B. breve* 110^{1A}), P-B+ (1622) (*B. bifidum* 162^{2A}) and P + B+ (1622) (microbial consortium + *B. bifidum* 162^{2A}). **(A)** Representative images of alveolar bone. All data were obtained in the region between the red points. Data on Alveolar bone volume (ABV) (Average and sd) determined in pixels^3 **(B)**, Percentage of alveolar bone volume (Average and sd) and **(C)** Percentage of total porosity (Average and sd) **(D)** of the different groups. * Statistically significant difference in relation to negative control (SHAM), # Statistically significant difference in relation to positive control (P + B-). ANOVA, Tukey's multiple comparison, p <0.05%. Data representative of two independent experiments (n = 8 mice/per group).

in gingival tissues of groups P + B- and negative control (SHAM). Treg cells populations were also similar in all experimental groups (**Figure 4**). The administration of *B. bifidum* 162^{2A} did not induce changes in Th17 cells population. However, the oral administration of *B. breve* 110^{1A} increased $CD45^+CD3^+CD4^+ROR\gamma t^+$ Th17 cells population from 0.31% in SHAM gingival tissue samples to 1.79% in P + B+ (1101) and 3% in P-B+ (1101), indicating that oral inoculation of *B. breve* 110^{1A} induces a Th17 response (**Figure 5**).

Serum Cytokines Levels

Serum levels of IL-10 were similar in groups P + B- and negative control (SHAM). However, the bifidobacteria oral regimen decreased serum levels of IL-10, especially in the groups that were also inoculated with the microbial consortium (**Figure 6**). There were no differences in TNF-α levels among groups (data not shown).

DISCUSSION

In the present study, the infection model with oral inoculation of *P. gingivalis, F. nucleatum*, *P. intermedia* and *S. gordonii* was efficient in reducing alveolar bone volume and increasing total bone porosity in C57Bl/6 mice. Thus, the protocol was able to induce experimental periodontitis, as shown in other studies (Blasco-Baque et al., 2012; Barbosa et al., 2015; Kuboniwa et al., 2017). Two strains of *P. gingivalis* were used, since multiple strains of the pathogen showing different virulence strategies occur in humans (Amano et al., 2000). Despite bone resorption, *P. gingivalis* was not detected at the end of the experimental period, and expression of *Il-1β*, *Tnf-α*, and genes encoding receptors to PAMPS, as well as the percentage of Treg and Th17 cells populations were not altered in gingival samples of the periodontitis group (P + B-) when compared to SHAM. Some studies demonstrated that the inflammatory cytokines were detected only in the initial phase of induction of periodontitis in mouse model (Polak et al., 2009; Ebbers, et al., 2018), thus for cytokine detection, early time points analysis would be required. In contrast, expression of *Il-17* was downregulated by the microbial consortium, confirming the breakage of homeostasis. These findings are in accordance with others who reported that persistent colonization of *P. gingivalis* may not be achieved, but bone loss is seen due to dysbiosis promoted by *P. gingivalis*, a keystone pathogen (Payne et al., 2019). Similarly, *P. gingivalis* strain W83 does not up-regulate transcription of *Tnf-α* and *Il-1β* in mice gingival tissues (Sato et al., 2018). Moreover, the effects of administration of bifidobacteria differed in mice challenged with the microbial consortium from control mice.

We have shown that the oral administration of *B. bifidum* 162^{2A}, but not of *B. breve* 110^{1A}, was able to control alveolar bone loss induced by the microbial consortium. These *in vivo* data contrast to *in vitro* results showing that *B. breve* 110^{1A} and *B. bifidum* 162^{2A} can aggregate in multispecies biofilms formed by *P. gingivalis, S. oralis* and *S. gordonii*, and reduce the abundance of *P. gingivalis* without affecting the abundance of early colonizers

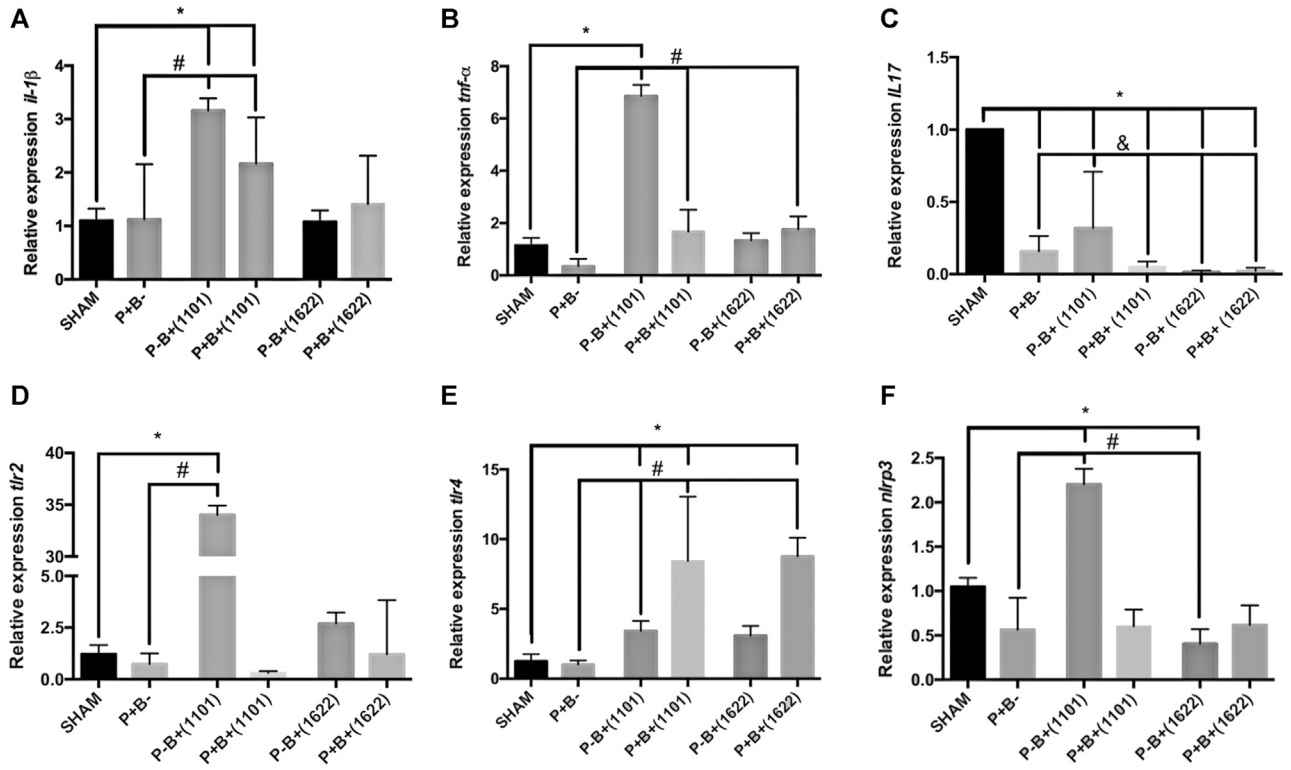

FIGURE 3 | Bifidobacteria alter transcription of genes encoding cytokines and receptors for PAMPS in gingival tissues. Relative transcription of *Il-1β* **(A)** and *Tnf-α* **(B)**, *Il-17* **(C)**, *Tlr2* **(D)**, *Tlr4* **(E)** and *Nrlp3* **(F)**, determined by RT-qPCR in gingival tissues of C57Bl/6 mice submitted to different treatments for 45 days of experimental period: SHAM (negative control), P + B- (positive control), P-B + (1101) (*B. breve* 110^{1A}), P + B+ (1101) (microbial consortium + *B. breve* 110^{1A}), P-B+ (1622) (*B. bifidum* 162^{2A}) and P + B+ (1622) (microbial consortium + *B. bifidum* 162^{2A}). * Statistically significant difference in relation to negative control (SHAM), # Statistically significant difference in relation to positive control (P + B-). & Statistically significant difference in relation to P-B+ (1101). ANOVA, Tukey's multiple comparison, p <0.05%. Data representative of two independent experiments (n = 8 mice/per group).

(Ishikawa et al., 2020). The beneficial effect of *B. breve* was also suggested by a strong antioxidant capacity of *B. breve* strain A28, which protects host cells against reactive oxygen species produced during the inflammatory process (Mendi and Aslım, 2014). However, this feature is not homogenous within this specie, and the antioxidant capacity of the studied bifidobacteria was not determined.

The benefit of bifidobacteria species in the control of periodontitis has been previously shown for *B. animalis subsp. lactis* HN019. The topical use of this strain prevented alveolar bone loss in rats submitted to ligature induced periodontitis (Oliveira et al., 2017). Another animal study reported the beneficial effect of *B. animalis subsp. lactis* HN019 even as adjunct to scaling and root planning (SRP), with concomitant reduction in the number of osteoclasts, decrease in *IL-1β* transcripts and increased expression of *IL-10* in the periodontal tissues (Ricoldi et al., 2017). Similarly, the adjunctive use of *Bifidobacterium animalis subsp. lactis* HN019 in humans to treat periodontitis improved periodontal clinical parameters such as probing pocket depth and clinical attachment gain, and reduced the colonization levels of *P. gingivalis*, *Treponema denticola* and other pathobionts (Invernici et al., 2018).

Herein, a distinct response to different *Bifidobacterium* species was not only seen in terms of alveolar bone loss. The groups receiving each bifidobacteria strain (P-B+), or each bifidobacteria plus the microbial consortium (P + B+) differed also in other parameters such as weight gain, expression of cytokines and receptors in the gingival tissue, and Th17 cells percentage.

B. breve 110^{1A} lack of effect in preventing alveolar bone loss induced by the periodontal pathogenic consortium was followed by lower weight gain and higher inflammatory response in gingival tissues when compared to the other groups. The inflammatory profile induced by *B. breve* 110^{1A} was evidenced by the upregulation of transcription of *Tnf-α*, *Il-1β*, *Tlr2*, *Tlr4*, and *Nrlp3*, a slight down-regulation of *Il-17* and increase of Th17 cells population in the gingival tissues. On the other hand, administration of *B. bifidum* 162^{2A} alone or in combination with the microbial consortium did not induce any changes, except for a substantial down regulation of *Il-17* mRNA levels and an upregulation of *TLr4* in mice challenged with the microbial consortium.

The oral administration of the two bifidobacteria also yielded different outcomes on the transcription of *Nrlp3* and *Il-1β*, suggesting their influence on inflammasome modulation. *B. breve* 110^{1A} up-regulated transcription of *Nrlp3* and *Il-1β*

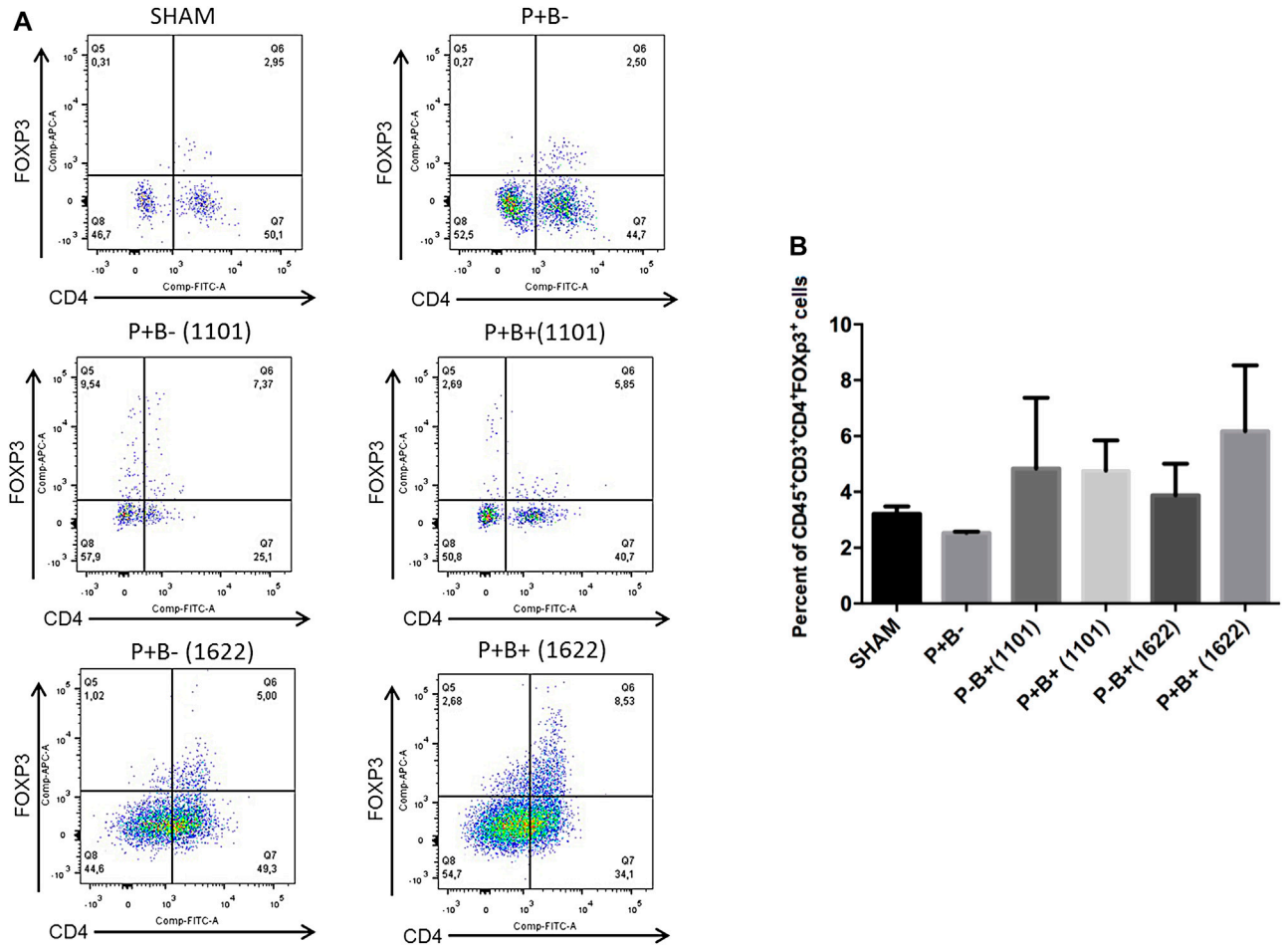

FIGURE 4 | Treg populations remained unchanged in gingival tissues of C57Bl/6 mice submitted to different treatments: SHAM (negative control), P + B- (microbial consortium), P-B+ (1101) (*B. breve* 110^{1A}), P + B+ (1101) (microbial consortium + *B. breve* 110^{1A}), P-B+ (1622) (*B. bifidum* 162^{2A}) and P + B+ (1622) (microbial consortium + *B. bifidum* 162^{2A}). In **(A)** representative flow cytometry diagram showing the gating of $CD4^+$, FoxP3+ Treg cells. In **(B)** Average percentages of $CD4^+$, FoxP3+ Treg cells. No differences among the groups. ANOVA, Tukey's multiple comparison, $p>0.05\%$. Facs plots represent the results of one of two independent experiments with similar results (n = 2 pooled samples from 4 mice/per group).

compared to the SHAM group, whereas *B. bifidum* 162^{2A} down-regulated *Nrlp3* mRNA levels and did not affect transcription of *Il-1β*. However, concomitant administration of the microbial consortium with the bifidobacteria resulted in decrease of the high mRNA levels of *Nrlp3* and *Il-1β* induced by *B. breve* 110^{1A} and an increase in these transcripts in the *B. bifidum* 162^{2A} group, although it did not reach the high levels achieved by *B. breve* 110^{1A}. Previous *in vitro* data may help explaining the different outcomes on the control of periodontitis by the two tested bifidobacteria. *P. gingivalis* W83 challenged gingival epithelial cells showed increased viability when co-infected with *B. bifidum* 162^{2A} but not with *B. breve* 110^{1A} (Albuquerque-Souza et al., 2019). On the other hand, co-culture of *P. gingivalis* ATCC 33277 challenged GECs with *B. breve* 110^{1A} resulted in high production of IL-1β and CXCL-8 differing from infected GECs co-cultured with *B. bifidum* 162^{2A} (Albuquerque-Souza et al., 2019).

It is well known that inflammasome activation differs according to the challenging bacteria species, target cells and environmental conditions including periodontal tissues (Taxman et al., 2012; Okano et al., 2018; Aral et al., 2021). Thus, the variable regulation of Nrlp3 and Il-1β induced by the two bifidobacteria may play a role on their effects in periodontal tissues. Tissue destruction in periodontitis is associated with the positive regulation of inflammasome-associated receptors such as NRLP3 (Xue et al., 2015) and production of IL-1β (Silva et al., 2015). Thus, induction of *Il-1β* and *Nrlp3* transcription in gingival tissues by *B. breve* 110^{1A} under a commensal microbiome is indicative of its pro-inflammatory activity. In contrast, *B. bifidum* 162^{2A} may partially surpass pathogen´s strategy to inhibit inflammasome activation in order to evade host defenses (Taxman et al., 2012; Okano et al., 2018) since its administration in the group receiving the microbial consortium attenuated *Nrlp3* down-regulation.

Administration of *B. breve* 110^{1A} without any other challenge up-regulated the transcription of *Tlr4* and *Tlr2*. Up-regulation of

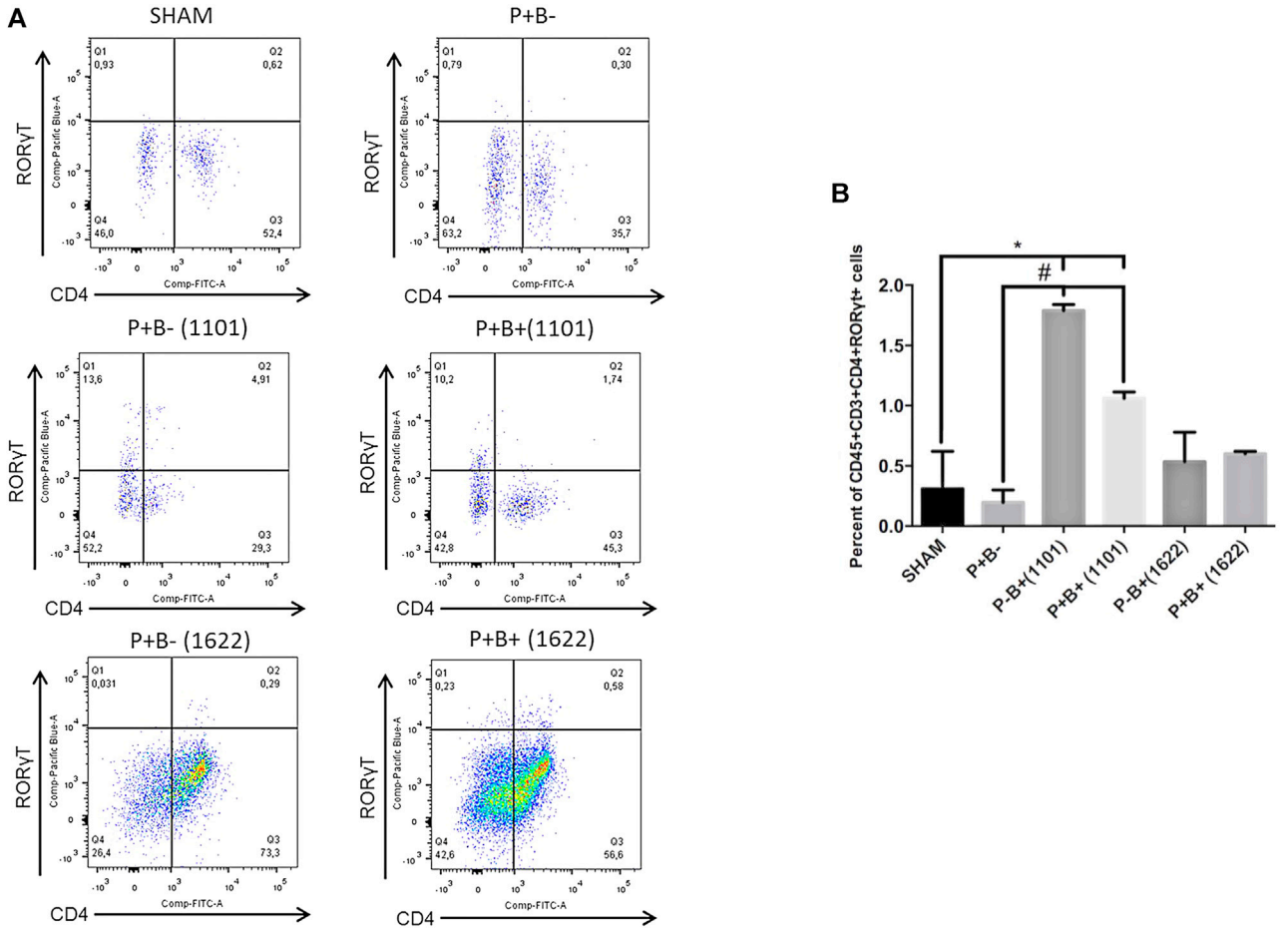

FIGURE 5 | Oral administration of *B. breve* 110^{1A} increases Th17 population in the gingival tissue. Th17 population in the gingival tissue of C57Bl/6 mice submitted to different treatments: SHAM (negative control), P + B- (microbial consortium), P-B+ (1101) (*B. breve* 110^{1A}), P + B+ (1101) (microbial consortium + *B. breve* 110^{1A}), P-B+ (1622) (*B. bifidum* 162^{2A}) and P + B+ (1622) (microbial consortium + *B. bifidum* 162^{2A}). In **(A)** representative flow cytometry diagram showing the gating of $CD4^+$, $ROR\gamma t^+$ Th17 cells. In **(B)** Average percentages of $CD4^+$, $ROR\gamma t^+$ Th17 cells. *Statistically significant difference in relation to negative control (SHAM), # Statistically significant difference in relation to positive control (P + B-). ANOVA, Tukey's multiple comparison, $p<0.05\%$. Facs plots represent the results of one of two independent experiments with similar results (n = 2 pooled samples from 4 mice/per group).

Tlr2 was previously shown for other probiotics (Li et al., 2019). *Tlr4* mRNA levels increased with concomitant administration of the microbial consortium and *B. breve* 110^{1A} or B. *bifidum* 162^{2A}. Bifidobacteria are known to upregulate *Tlr4* due to production of lactic acid (Kanmani et al., 2019). *B. breve* 110^{1A} induced upregulation of PRRs under a symbiotic microbiome suggests increased recognition of commensals, and pro-inflammatory down-stream cascades, as indicated by increased expression of *Tnf-α* and *Il-1β* in the P-B+1101 group. However, *Tlr4* up-regulation promoted by both *Bifidobacterium* under pathogens challenge may increase pathogen's recognition, and elimination. In contrast, down-regulation of *Tlr2* under *P. gingivalis* challenge may be beneficial, since TLR2 signaling in immune cells impairs their phagocytic activity, which promotes pathogen`s survival (Maekawa et al., 2014).

Regulatory T lymphocytes (Tregs) play a fundamental role in the control of inflammatory response, suppressing the proliferation and cytokine production of effector T cells, especially Th1 and Th17 (Gonzales, 2015). However, Treg populations in the gingival tissues of mice were not altered by the different treatments, suggesting this mechanism is not induced by the studied bifidobacteria. Oral inoculation of *B. breve* 110^{1A} altered T cells population in gingival tissues, leading to increased percentages of Th17, which was partially attenuated by the microbial consortium. Transcription of *Il-17* was demonstrated in the gingival tissue of the non-infected control mice, but all treatments with the microbial pathogenic consortium and/or the bifidobacteria down-regulated *Il-17* transcript levels. However, *Il-17* mRNA levels were higher in the group receiving *B. breve* 110^{1A}, whereas *B. bifidum* 162^{2A} induced the lowest levels among all groups. The inflammatory functions of Th17 cells depend on the different combinations of cytokines expressed in the environment (Bunte and Beikler, 2019) and these cells present multiple functions. While IL-17 production is key to homeostasis, Th17 exacerbated activation by microbial challenge can be deleterious (Moutsopoulos et al.,

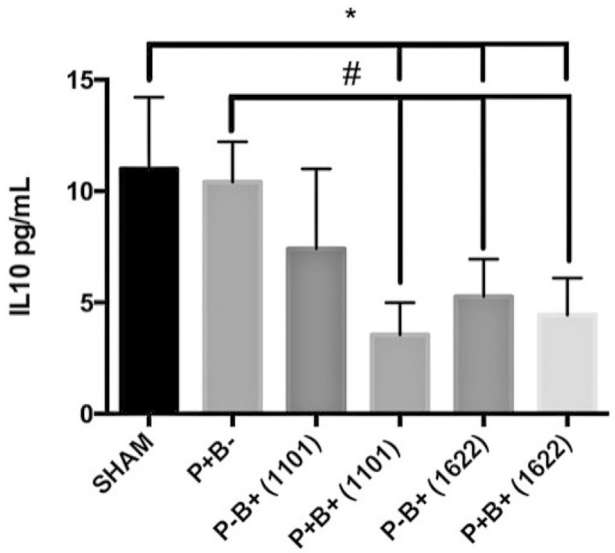

FIGURE 6 | Serum levels (pg/ml) of IL-10 in C57Bl/6 mice submitted to different treatments for 45 days: SHAM (negative control), P + B- (positive control), P-B+ (1101) (*B. breve* 110^{1A}), P + B+ (1101) (microbial consortium + *B. breve* 110^{1A}), P-B+ (1622) (*B. bifidum* 162^{2A}) and P + B+ (1622) (Microbial consortium + *B. bifidum* 162^{2A}). * Statistically significant difference in relation to negative control (SHAM), # Statistically significant difference in relation to positive control (P + B-). ANOVA, Tukey's multiple comparison, p <0.05%. Data representative of two independent experiments (n = 8 mice/per group).

2012). Thus, Th17 cell analyses also indicated that *B. breve* 110^{1A} induces an inflammatory profile in gingival tissues, differing from *B. bifidum* 162^{2A}.

These data should be taken under the limitations of the mice model where *P. gingivalis* persistent colonization was not achieved. Furthermore, the data were obtained at a single time point, 12-days after the last inoculation of the microbial consortium, whereas bifidobacteria were administered throughout the 45-days experimental period. It is also worthy to emphasize that the immune cells evaluation was performed with only two pooled samples per group, to minimize the number of mice, but the data were reproducible in two independent assays.

Overall, our data showed that the beneficial effect of *Bifidobacterium* to the periodontal tissues is not a common trait of this genus. The pro-inflammatory effect of *B. breve* 110^{1A} in gingival tissues may indicate increased defenses against invading organisms but also brings some concern on the safety of this species. Our data are corroborated by others reporting bacteremia by bifidobacteria, including *B. breve* (Esaiassen et al., 2017), even when used as a probiotic (Sato et al., 2016). Therefore, considering that probiotics outcomes depend on factors related to the host and their microbiome, their outspread use should be rethought. On the other hand, *B. bifidum* 162^{2A} is a potential candidate as a probiotic to control periodontitis. *B. bifidum* 162^{2A} did not lead to significant changes in inflammatory parameters and prevented alveolar bone loss without noticeable side effects. Further studies are still needed before its clinical indication.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (ICB USP Approval number: 1112017).

AUTHOR CONTRIBUTIONS

Conceptualization, MPAM draw the project, planned the assays, and wrote the manuscript. NOSC and MRLS draw the project and planned the assays. NS, KI and AC planned and ran the assays. JN isolated and CF characterized the Bifidobacteria strains. DK, EAS, ESA-S, MB, LS and BO helped with the animal model and samples collection. JL helped with the flow cytometer assays. MRM and PS helped with the microtomography analyses. All authors revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2021.713595/full#supplementary-material

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Insight Into the Beneficial Role of *Lactiplantibacillus plantarum* Supernatant Against Bacterial Infections, Oxidative Stress, and Wound Healing in A549 Cells and BALB/c Mice

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Lactiplantibacillus plantarum MTCC 2621 is a well-characterized probiotic strain and is reported to possess many health benefits. However, the wound healing potential of this probiotic is yet to be explored. Here, we have assessed the antibacterial, antioxidant, and wound healing activities of cell-free supernatant of *Lactiplantibacillus plantarum* MTCC 2621 (Lp2621). Lp2621 exhibited excellent antibacterial activity against the indicator bacteria in the agar well diffusion assay. Lp2621 did not show any hemolytic activity. The safety of Lp2621 gel was established using the skin irritation assay in BALB/c mice, and no dermal reactions were observed. The supernatant showed 60–100% protection of A549 cells against H_2O_2-induced stress. In the scratch assay, Lp2621 accelerated wound healing after 24 h of treatment. The percent wound healing was significantly higher in cells treated with Lp2621 at 18–24 h posttreatment. In an excision wound healing in mice, topical application of Lp2621 gel showed faster healing than the vehicle- and betadine-treated groups. Similar wound healing activity was observed in wounds infected with *Staphylococcus aureus*. Histological examination revealed better wound healing in Lp2621-treated mice. Topical treatment of the wounds with Lp2621 gel resulted in the upregulation of pro-inflammatory cytokine IL-6 in the early phase of wound healing and enhanced IL-10 expression in the later phase. These findings unveil a protective role of Lp2621 against bacterial infection, oxidative stress, and wound healing.

Keywords: oxidative stress, wound healing, inflammation, cell-free supernatant, A549 cells, BALB/c mice, *Lactiplantibacillus plantarum*

1 INTRODUCTION

Wound healing is a multifaceted biological process involving many extracellular and intracellular macromolecules. Healing occurs in four steps: hemostasis, inflammation, proliferation, and maturation (Vaid et al., 2020), and has a vital role in skin remodeling after injury (Mousavi et al., 2020). The economic evaluation of chronic wounds suggests that care and treatment of the wound are time-consuming and cost billions of dollars every year (Nussbaum et al., 2018). Although antibiotic therapies are in place for routine care and management of the wound, they do not cover all characteristics of wound management (Nussbaum et al., 2018). Thus, researchers and the scientific community have focused their efforts on developing an alternative strategy of using probiotics that aids in the wound healing process. The World Health Organization (WHO) defines probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2002). Probiotics lower the risk of infectious diseases, and in combination with antibiotics, combat secondary infections (King et al., 2014) as well as reduce the incidence and severity of diarrhea associated with antibiotic therapy (Hempel et al., 2012). They primarily belong to the genus *Lactobacillus* and *Bifidobacterium* (Soccol et al., 2010), and effectively modulate the immune function of the host by maintaining the balance of the intestinal microbiota (Ouwehand et al., 2016), improve the innate immunity, and moderate the functions of dendritic cells, macrophages, and T and B lymphocytes (Georgieva et al., 2015). In addition, they have also been shown to promote wound healing and modulate the inflammation caused by the pathogens through the toll-like receptor-controlled pathways (Vanderpool et al., 2008; Gholami et al., 2020). Studies have revealed that the direct application of lactic acid bacteria (LAB) on injured skin may improve skin health and augment its capacity to fight against various diseases (Nole et al., 2014; Knackstedt et al., 2020). Certain strains of the *Lactobacillus* genus play an important role in the wound healing process and protect the skin against inflammation and infections by the competitive inhibition of pathogens for adhesion sites and nutrients, modulation of the host immune response, and production of cytokines and secondary metabolites such as short-chain fatty acids as well as antimicrobial peptides (Halper et al., 2003; Peral et al., 2009; Sonal Sekhar et al., 2014; Lukic et al., 2017; Ong et al., 2020). The strain *Lactobacillus plantarum* MTCC 2621 {now renamed as *Lactiplantibacillus plantarum* [*Lpb. plantarum*] (Zheng et al., 2020)} used in this study (equivalent to *Lpb. plantarum* ATCC 8014) has been characterized for its probiotic properties (Tambekar and Bhutada 2010; Sreevani and Kumari 2013; Pop et al., 2016; Malakar et al., 2017; Khalil et al., 2018; Monteiro et al., 2019). *Lpb. plantarum* MTCC 2621 exhibited immunomodulatory activity *via* the downregulation of pro-inflammatory cytokines (Goad et al., 2013), whereas competitive inhibition with pathogens and the production of antimicrobial agents resulted in the pro-fertility property (Bhandari and Prabha 2015). The beneficial roles of this probiotic have been reported in various diseases; however, the efficacy of L*pb. plantarum* MTCC 2621 has not yet been fully elucidated in wound healing. Therefore, in this study, we assessed the antibacterial, antioxidant, and wound healing properties of the cell-free supernatant of *Lpb. plantarum* MTCC 2621 (henceforth read as Lp2621) using A549 cells *in vitro* and in a mouse model of wound healing.

2. MATERIALS AND METHODS

2.1 Chemicals

De Man, Rogosa, Sharpe MRS agar, MRS broth, and nutrient agar were purchased from HiMedia, and Triton X-100; carboxymethyl cellulose, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were purchased from Sigma. The reagents for cell culture media, RPMI, and fetal bovine serum (FBS) were obtained from GIBCO. H_2O_2 used to induce oxidative stress was purchased from Merck.

2.2 Cell Line

A549 cell line (human lung carcinoma cell line) was purchased from the National Centre for Cell Science (NCCS), Pune, India.

2.3 Bacterial Cell Culture

Lactiplantibacillus plantarum MTCC 2621, *Staphylococcus aureus* MTCC 737, *Micrococcus luteus* MTCC 106, *Pseudomonas aeruginosa* MTCC 1934, *Bacillus subtilis* MTCC 441, *Escherichia coli* MTCC 739, and *Klebsiella pneumoniae* MTCC 618 were obtained from the Microbial Type Culture Collection (MTCC) (CSIR-Institute of Microbial Technology, Chandigarh, India). The culture of *Lpb. plantarum* was grown in MRS broth at 37°C with 1% (v/v) inoculum. The culture was centrifuged at 5000 rpm for 10 min, and the cell-free supernatant was collected and used in this study. All other strains were grown on the nutrient agar media at 37°C for 24 h.

The flowchart of the experimental design of the study is illustrated in **Figure 1**.

2.4 *In Vitro* Studies

2.4.1 Antibacterial Activity of Lp2621

The antibacterial property of Lp2621 was determined using the agar well diffusion assay as per previous studies (Dahiya and Purkayastha 2012). Six indicator strains were used viz. *S. aureus* (MTCC 737), *M. luteus* (MTCC 106), *P. aeruginosa* (MTCC 1934), *B. subtilis* (MTCC 441), *E. coli* (MTCC 739), and *K. pneumoniae* (MTCC 618). MRS broth alone was used as the negative control.

2.4.2 Hemolytic Assay for Toxicity Testing of Lp2621

Blood collected from New Zealand white rabbit was centrifuged, washed three times with 1X PBS, and resuspended in phosphate-buffered saline (PBS) at a concentration of 4% (vol/vol). The hemolytic assay was performed as described by Jangra et al. (2019) using different concentrations of Lp2621 (0.78–100 percent in two fold dilutions). Triton X-100 (0.1%), MRS broth, and PBS were used as positive, vehicle, and negative controls, respectively.

Percent hemolytic activity was calculated using a formula.

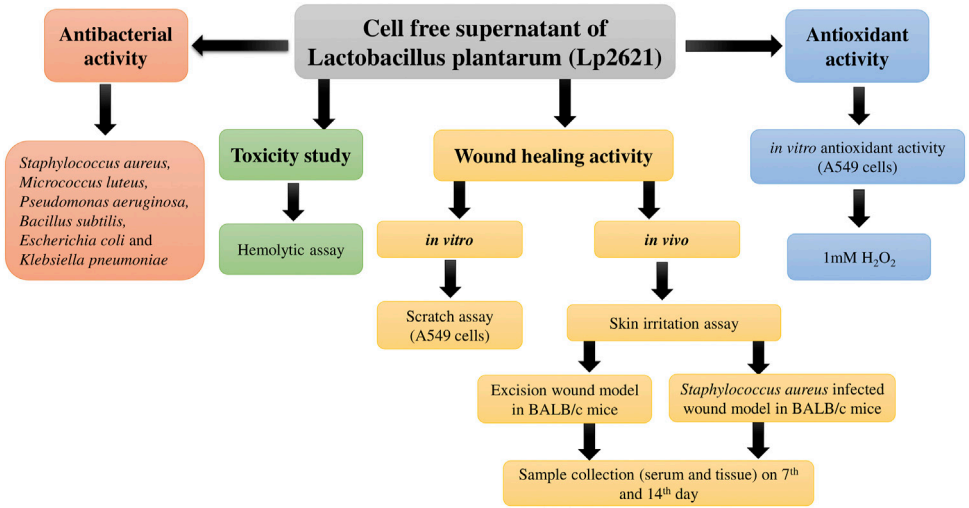

FIGURE 1 | Flowchart depicting the study design for evaluation of antibacterial, antioxidant, and wound healing potential of Lp2621.

$$\% \text{ Hemolysis} = \frac{\text{Absorbance of Test} - \text{Absorbance of Blank}}{\text{Absorbance of positive control} - \text{Absorbance of Blank}} X\ 100. \quad (1)$$

2.4.3 Scratch Assay to Evaluate the Wound Healing Ability of Lp2621

The scratch assay was carried out according to Vaid et al. (2020), with slight modifications. A549 cells (1×10^6 cells/well) were grown in the RPMI culture medium with 10% FBS in six-well plates and incubated overnight at 37°C in a humidified CO_2 incubator. After incubation, the medium was removed completely, and a scratch was created on the adherent cell layer in each well by using a sterile 200 µl pipette tip. The wells were washed with 1X PBS to remove cellular debris. The RPMI medium having Lp2621 (grown up to 12 h) at a dose of 12.5 and 6.25% was added to respective wells. The positive and solvent controls received RPMI supplemented with 10% FBS and RPMI with 10% FBS and 12.5% MRS broth (used to prepare Lp2621), respectively. In negative control wells, only the RPMI medium was added. Photographs of the scratch area (wound area) were captured at 0, 6, 12, 18, and 24 h by using a trinocular microscope having a in-built camera. Data were evaluated to calculate the percent wound area using ImageJ software (LOCI, the University of Wisconsin).

2.4.4 Beneficial Role of Lp2621 on H_2O_2-Induced Oxidative Stress in A549 Cells

Antioxidant activity of Lp2621 was evaluated by an assay in which oxidative stress was induced in A549 cells by H_2O_2 and the cell viability was evaluated by MTT assay according to Vaid et al. (2020), with slight modifications. Various concentrations of Lp2621 (0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, and 100%) diluted in RPMI having 10% FBS and 1% pen-strep were used to treat cells in different protocols as mentioned below:

a. Concomitant exposure of A549 cells to both Lp2621 and 1.0 mM H_2O_2 for 24 h.
b. Exposure of A549 cells to 1.0 mM H_2O_2 for 4 h followed by Lp2621 treatment for 24 h.
c. 24 h pretreatment of the A549 cells with Lp2621 followed by exposure of 1.0 mM H_2O_2 for 4 h.

2.5 Preparation of Gel Containing Lp2621

Lpb. plantarum (MTCC 2621) was cultured in MRS broth at 37°C. The culture with 1×10^9 CFU/ml was centrifuged at 5000 rpm for 10 min, and the cell-free supernatant was collected. The gel was formulated by adding 2% carboxy methyl cellulose (CMC) to the supernatant and was mixed thoroughly at room temperature until the uniform gel was formed, and stored at 4°C for further use.

2.6 *In Vivo* Analysis

2.6.1 Animals

Eight-week-old BALB/c mice (19–25 gm weight) were taken from the IMTECH Centre for Animal Resources and Experimentation (iCARE) facility of the institute. Mice were housed in individually ventilated cages under controlled conditions of temperature (24–25°C), light (photoperiod of 12:12), and humidity (30–70%), and were provided pelleted diet and water *ad libitum*. Before starting the experiment, randomization of the animals was done, and the mice were left for a week prior to the experiment for acclimatization. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC) of CSIR-Institute of Microbial Technology (IAEC approval number IAEC/20/18) and performed as per the principles and guidelines of the Committee for the Purpose of Supervision of Experiments on Animals (CPCSEA), Ministry of Fisheries, Animal Husbandry and Dairying, India.

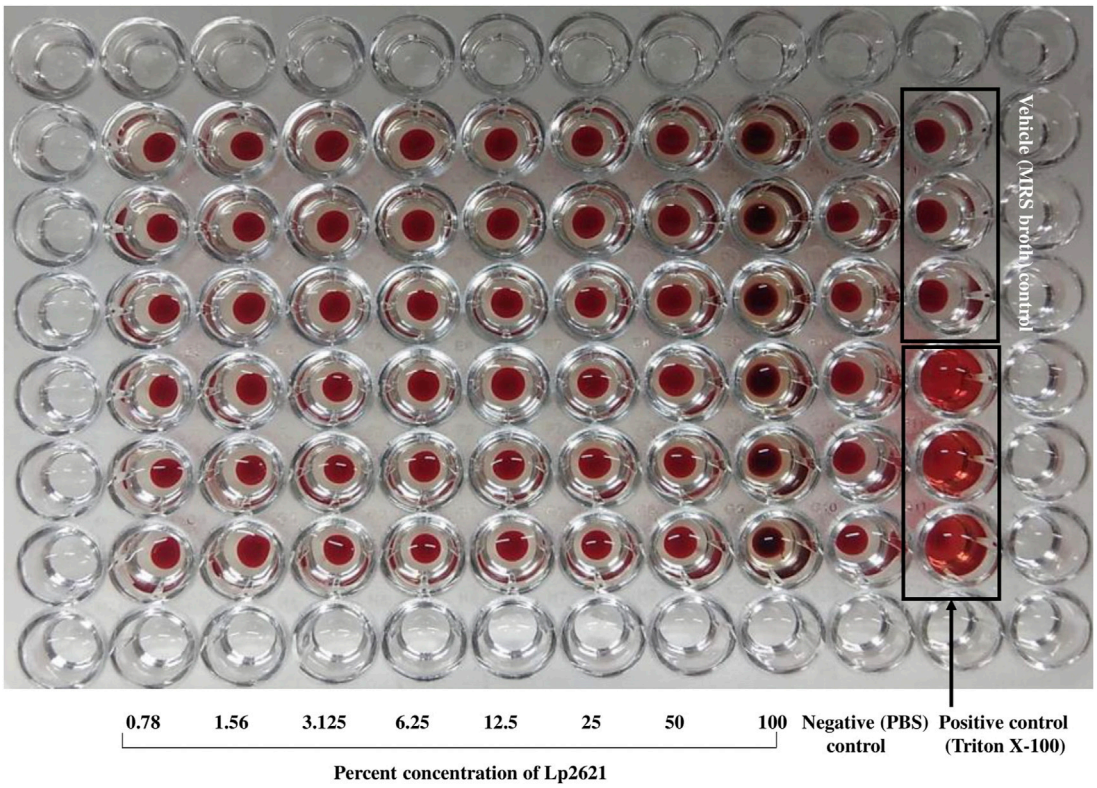

FIGURE 2 | Hemolytic property of Lp2621. The image is a representative of two independent experiments performed in triplicates.

2.6.2 Skin Irritation Assay

Skin irritation assay was performed to evaluate the safety of Lp2621 gel in BALB/c mice (Draize et al., 1944). The dorsal back of the mice was shaved to remove hair, without damaging the skin surface, 24 h before the assay. The mice were divided into three groups (N = 3) according to the treatment plan, and each group was having three mice: Group I: 20% sodium lauryl sulfate (SLS) solution (positive control), Group II: CMC gel (negative control), and Group III: gel containing Lp2621 and housed individually. The gel was applied topically to the shaved skin area (approximately 1 cm^2), and the applied sites were observed for any dermal reactions such as erythema and edema at 24, 48, and 72 h post-application. The mean erythema and edema scores were recorded based on their degree of severity caused by the application of gel as follows: no erythema/edema = 0, slight erythema/edema = 1, moderate erythema/edema = 2, and severe erythema/edema = 3.

2.6.3 Wound Healing Activity of Lp2621

Having shown the antibacterial activity in the agar well diffusion assay and the wound healing activity of Lp2621 in a scratch assay using A549 cells, we next planned to conduct two experiments on the mice to check whether Lp2621 gel would be effective in wound healing and/or treating wounds infected with *S. aureus* infection as well. The mice were anesthetized using isoflurane (gas anesthesia). The hair on the dorsal side of the skin was removed, and the area was cleaned and disinfected using 70% ethanol. A full-thickness excision wound of 8 mm diameter was created in the skin of the dorsal part of mice with a sterile biopsy punch. The mice were randomized into three groups (N = 3) having nine mice in each group (n = 9) viz. vehicle control (CMC), positive control (betadine), and Lp2621 (test group). Each group of mice received topical application of the respective treatment twice a day for 21 days. The study continued up to 21 days, the images of the wounds were taken at days 0, 7, 14, and 21 of the study, and the wound area was calculated using ImageJ software. The percent wound contraction was calculated by the following formula.

$$\text{Percent wound contraction} = \frac{\text{Healed area}}{\text{Total area}} \text{X } 100. \qquad (2)$$

Three mice were euthanized at days 7 and 14 after treatment from each group. In the sham control group (n = 3), a wound was created without applying any treatment, and mice were euthanized after 24 h. Wound tissues were collected from different groups of mice and fixed in 10% neutral buffer formalin (NBF) solution for histopathological studies. Blood was collected from mice of different groups, and the serum was isolated and stored at −80°C for cytokine analysis.

2.6.4 Efficacy of Lp2621 Gel on an Excision Wound Healing Model Infected With *S. aureus*

The same procedure for induction of a full thickness excision wound in the skin of the dorsal part of the mice was followed as mentioned under 2.6.3. Mice (n = 9) were divided into three groups. The grown *S. aureus* culture was centrifuged at 5,000 g for 10 min, the media

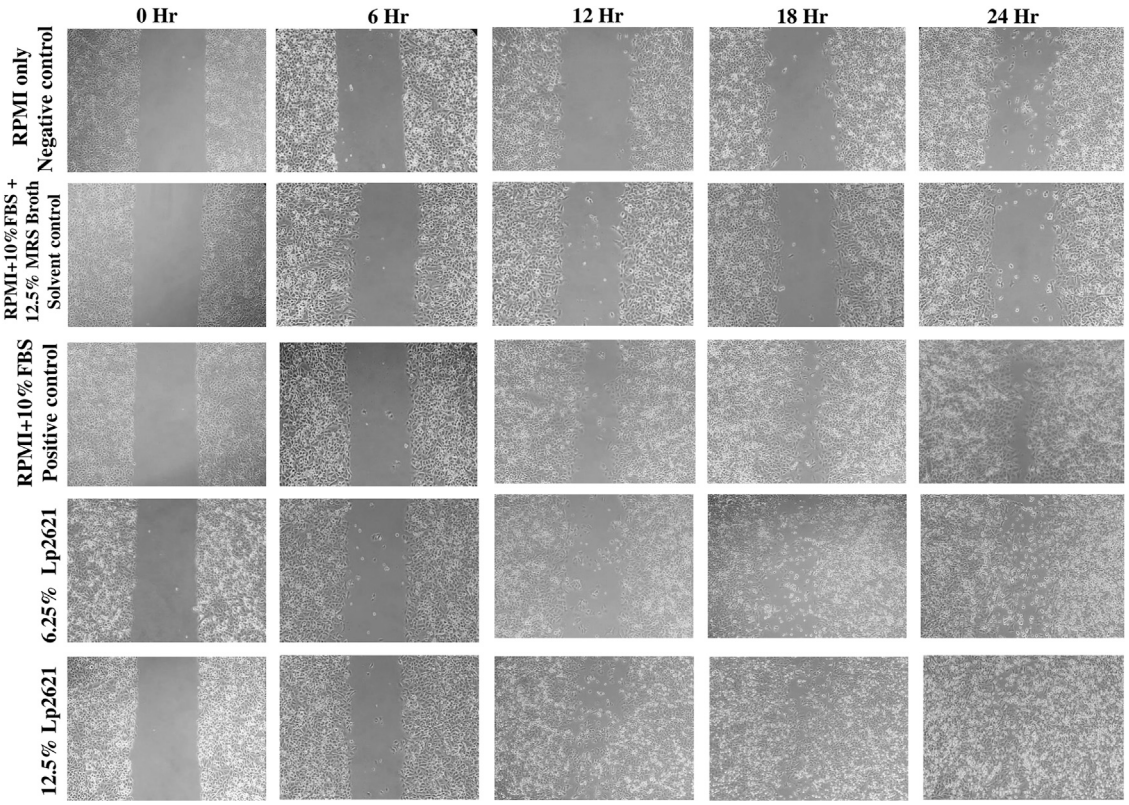

FIGURE 3 | Representative microscopic images of the wound area in A549 epithelial cells in scratch assay after 0, 6, 12, 18, and 24 h incubation. Images were taken using a trinocular microscope having an in-built camera. Analysis was done using ImageJ software.

was discarded, and the pellet was washed twice with PBS. Bacterial infection was initiated by placing a droplet containing 10^7 CFU cells on the excision wound as created earlier. The treatment of the infectious wound was started 4 h postinfection. Group I was treated with CMC as the negative control, group II with betadine, and group III with Lp2621 gel. The gross images of wounds were recorded and analyzed to calculate the percent wound contraction as described above. The mice of each group ($n = 3$) were euthanized at days 7 and 14 of wounding. Blood samples and wound tissues were collected for analysis of pro- and anti-inflammatory cytokines in serum and histopathological examination, respectively.

2.6.5 Histopathology

Formalin-fixed wound tissues were processed and dehydrated with graded alcohol, cleared in xylene, and molded in paraffin. Sections of 4–5 μm thickness were prepared and stained with hematoxylin and eosin (H&E), and observed under a light microscope. The H&E staining was used to evaluate fibroblast proliferation, vascularization, re-epithelization, collagen deposition, granulation of tissue formation, and the infiltration of polymorphonuclear leukocytes (PMNL).

2.6.6 Cytokine Analysis

The cytokines (IL-6 and IL-10) were analyzed using a standard ELISA method. In a 96-well plate, primary antibodies, namely, IL-6 (2 μg/ml) and IL-10 (2 μg/ml), were coated in phosphate buffer (pH-9.2) and left overnight at 4°C. The next day after washing, sites were blocked with 1% bovine serum albumin for 2 h at 37°C. The plates were washed with phosphate buffer saline Tween-20, and pooled serum samples (50 μl) (dilution- 1:10) were added and incubated at 4°C overnight. Then, the biotinylated antibody was added in dilution buffer (1:1 solution of PBST and 1% BSA) and incubated at 37°C for 2 h. Streptavidin HRP (1:10,000) was added to each well, and the plate was incubated for 45 min at 37°C. Then the substrate OPD (o-phenylenediamine dihydrochloride)-H_2O_2 (1 mg/ml and 1 μl/ml) was added and observed for color development. The reaction was stopped using 7% H_2SO_4, and the reading was taken at 492 nm in an ELISA plate reader. After every step, washing was done with PBST.

2.7 Statistical Analysis

The results are expressed as the mean ± SE unless mentioned otherwise. All statistical analyses were done using the one-way analysis of variance (ANOVA) (SigmaPlot 11.0 program).

3. RESULTS

3.1 Antibacterial Activity

In the agar well diffusion assay, Lp2621 exhibited distinct zones of inhibition (in mm) against all tested indicator bacterial strains viz.

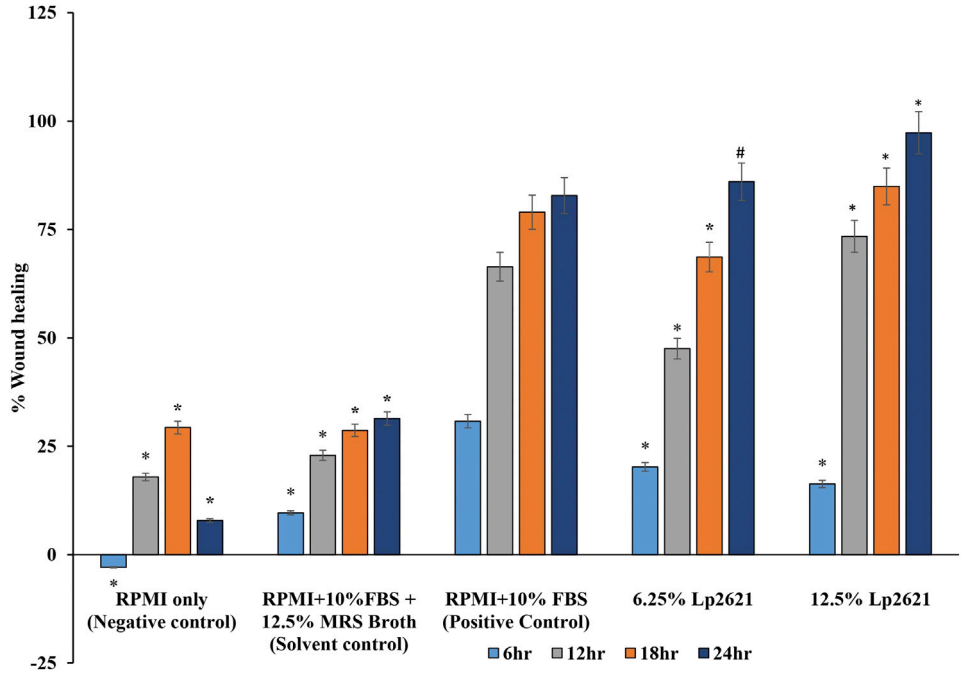

FIGURE 4 | Wound healing percent (%) in scratch assay after 6, 12, 18, and 24 h post-treatment with Lp2621. Data are representative of two independent experiments performed in triplicates and expressed as mean ± SE.*, and # mean $p < 0.001$ and $p = 0.003$.

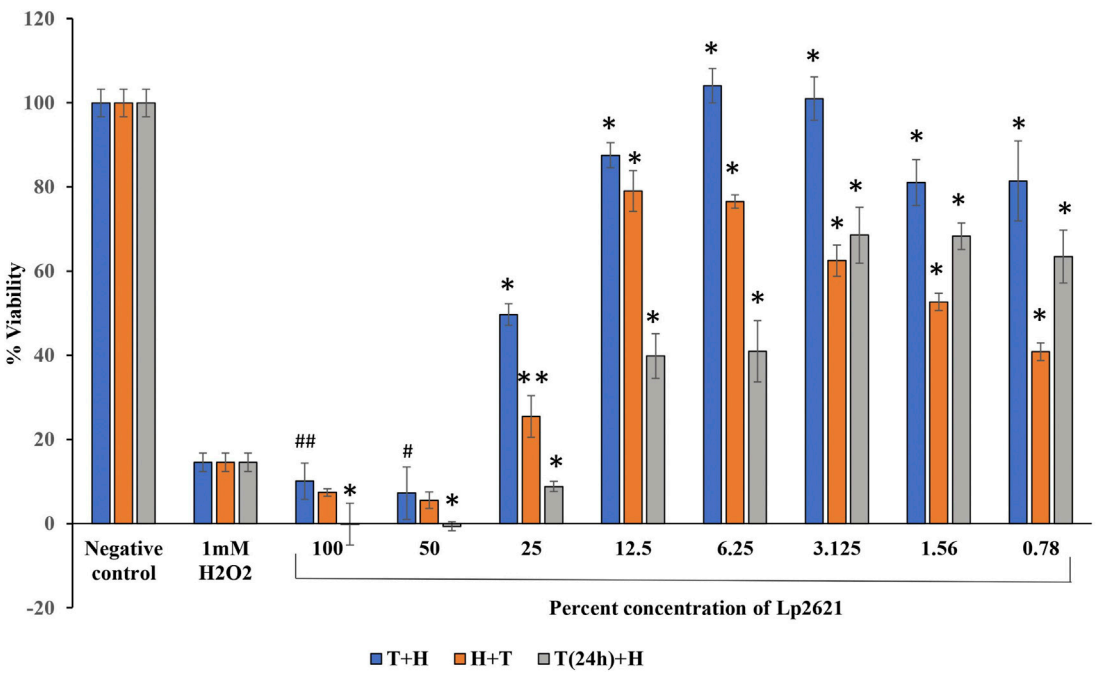

FIGURE 5 | Percent viability of A549 cells treated with Lp2621.

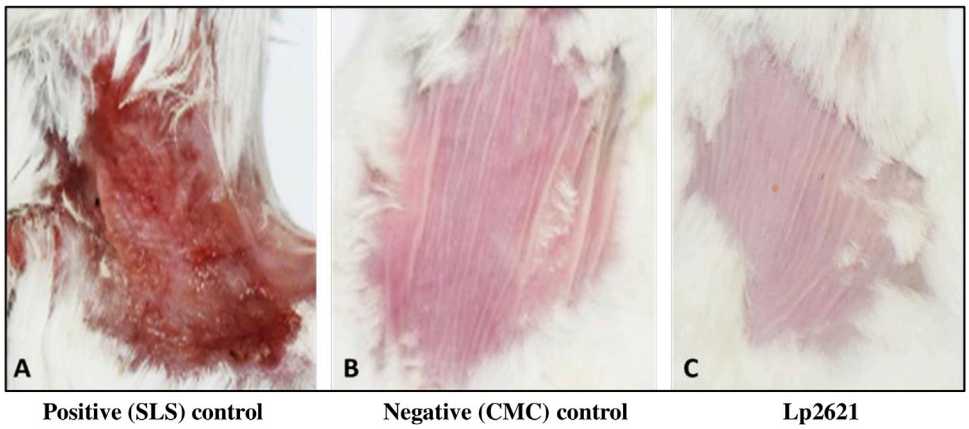

FIGURE 6 | Representative images of the skin irritation assay using Lp2621 at 72 h: **(A)** positive control, SLS treated, **(B)** negative control, CMC gel, and **(C)** treated with gel containing Lp2621.

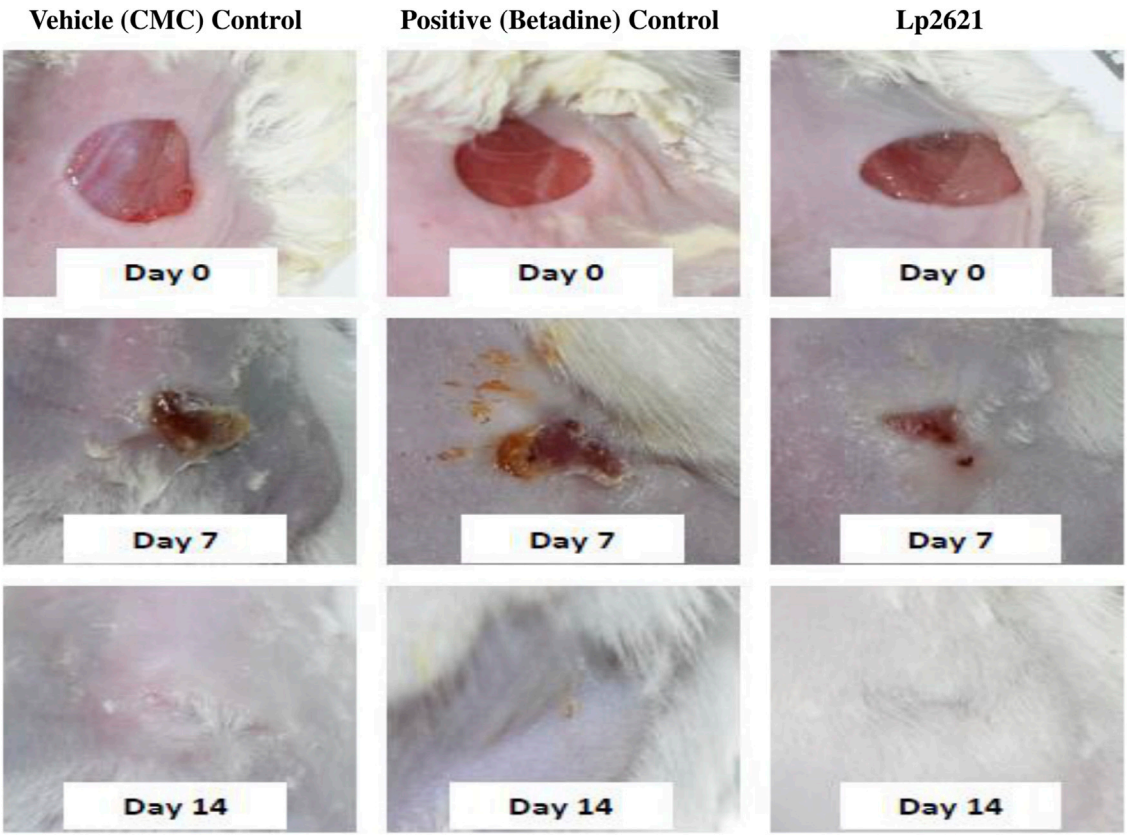

FIGURE 7 | Full-thickness excision wounds were created in mice. Representative photographs from the mice showing macroscopic wound closure on different day's post-injury.

S. aureus MTCC 737 (9.03 ± 0.39), *M. luteus* MTCC 106 (18.94 ± 0.31), *P. aeruginosa* MTCC 1934 (10.53 ± 0.59), *B. subtilis* MTCC 441 (10.48 ± 0.84), and *K. pneumoniae* MTCC 618 (8.82 ± 0.39), except *E. coli*.

3.2 Toxicity Test Using Hemolytic Assay

Lp2621 does not show any hemolytic activity at 0.78–100 percent concentration. In contrast, Triton X-100 (positive control) caused the complete lysis of RBCs (**Figure 2**).

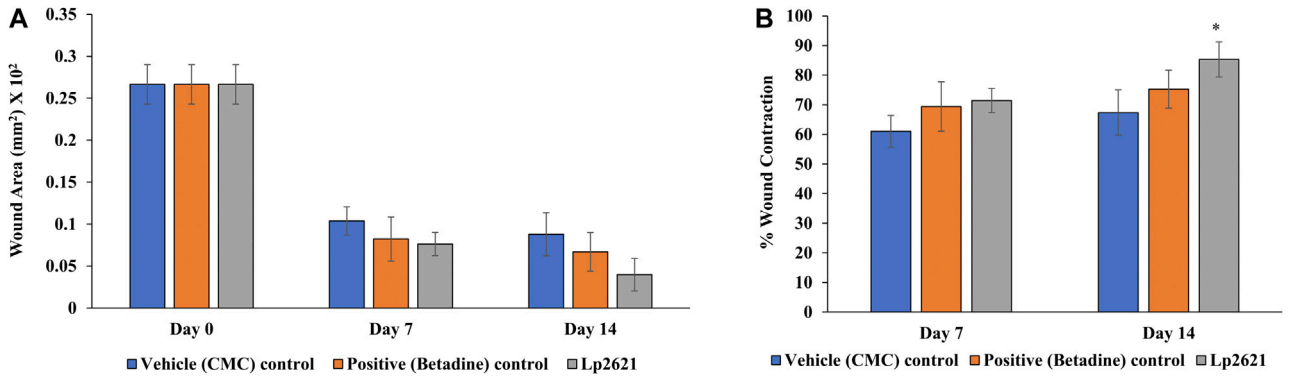

FIGURE 8 | Effect of topical treatment of Lp2621 gel; **(A)** wound area and **(B)** percent wound contraction at different day's post-wounding. Data are expressed as mean ± SD. *mean p = 0.033.

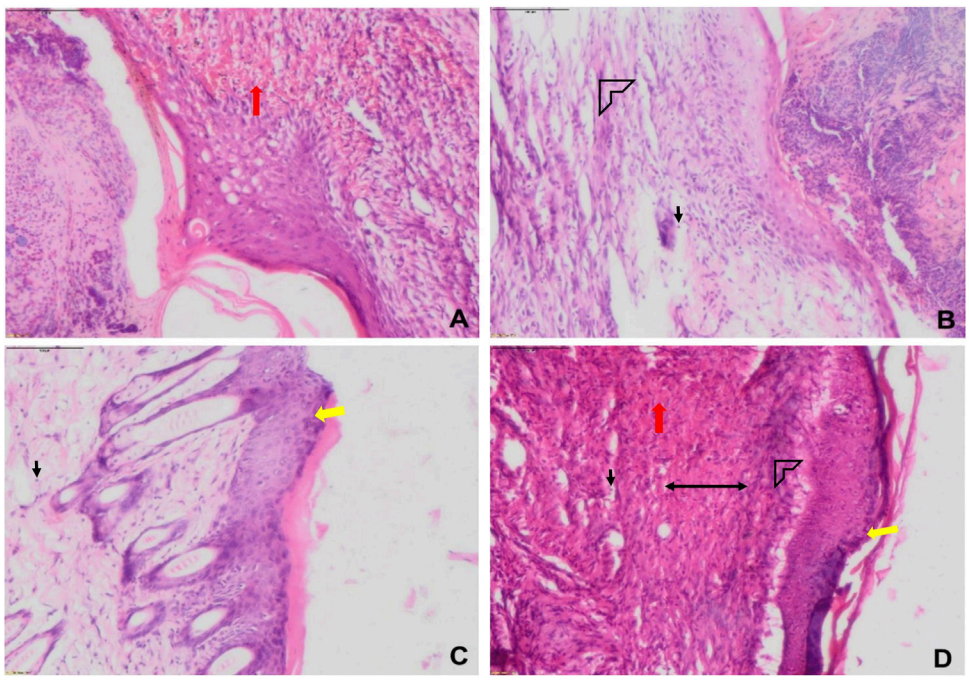

FIGURE 9 | Representative histological images of wounds of various groups on day 1 **(A)** and day 7 **(B–D)**. **(A)** sham, **(B)** vehicle (CMC), **(C)** positive (betadine), **(D)** Lp2621 (scale bar = 100 μm). Fibroblasts (black), vascularization (red), re-epithelization (yellow), collagen deposition (arrow head), and granulation of tissue (double-sided arrow).

3.3 Wound Healing Ability of Lp2621 in A549 Cells Using the Scratch Assay

The treatment of A549 cells with different concentrations (6.25 and 12.5%) of Lp2621 results in faster wound healing than the positive control after 24 h of treatment (**Figure 3**). Percent wound healing is significantly higher in cells treated with Lp2621 than in various controls (p < 0.001 and p = 0.003) at 18–24 h posttreatment (**Figure 4**).

3.4 Antioxidant Activity of Lp2621

Percent viability of A549 cells after treatment with Lp2621 and H_2O_2 is presented in **Figure 5**. Lp2621 exhibits 90–100% protection of the cells when treated at 12.5, 6.25, and 3.125% concentrations concomitantly with 1 mM H_2O_2 for 24 h (p < 0.001, p = 0.003 and p = 0.008). On the contrary, pre-exposure of cells to H_2O_2 for 4 h followed by treatment with Lp2621 at similar concentrations results in 60–80% cell viability (p < 0.001 and p = 0.001). Furthermore, 24 h pretreatment of cells with Lp2621 (3.125, 1.56, and 0.78%) followed by the exposure to H_2O_2 for 4 h results in a decline in cell viability to 60% (p < 0.001).

3.5 *In Vivo* Analysis

3.5.1 Skin Irritation Assay

Mice treated with negative control and gel containing Lp2621 did not display any abnormal irritation even after 72 h of application,

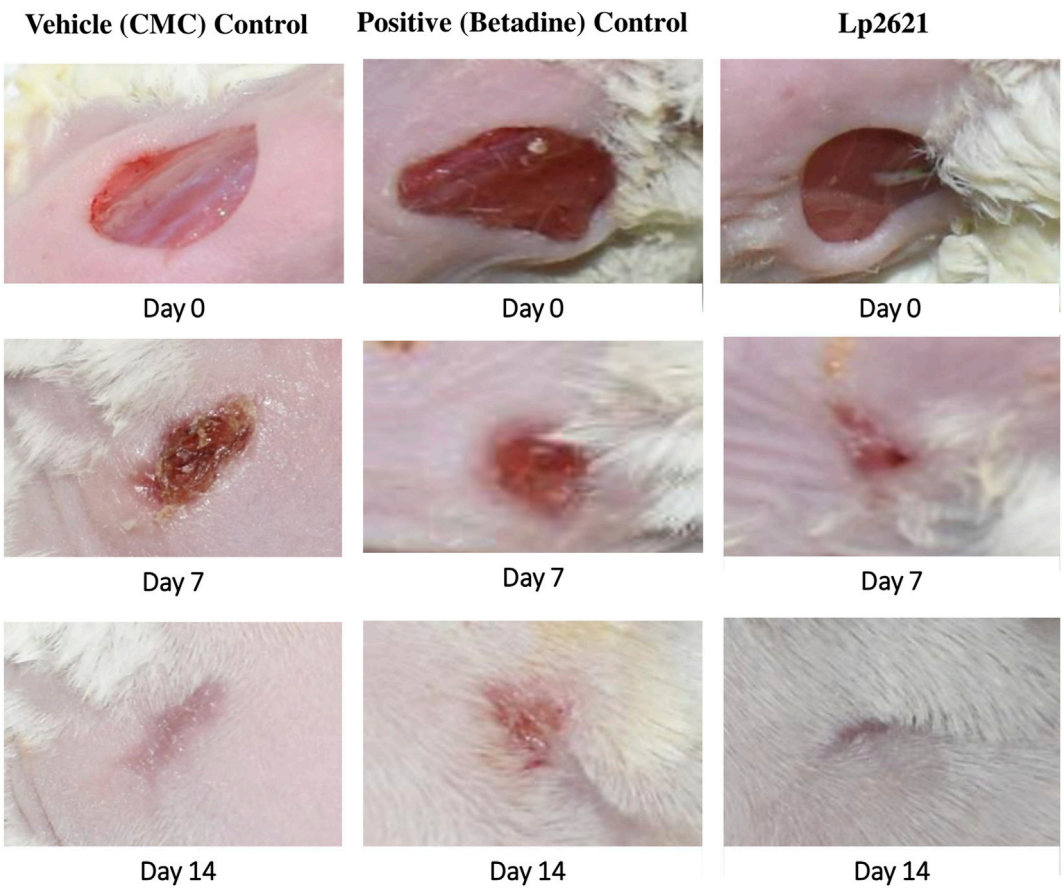

FIGURE 10 | Full-thickness excision wounds were created in mice and infected with S. *aureus*. Representative photographs from mice showing macroscopic wound closure on different day's post-injury.

whereas mice treated with SLS exhibits severe dermal reactions such as erythema and edema at the site of application (**Figure 6**).

3.5.2 Efficacy of Lp2621 Gel on Excision Wound Healing Model

The results showed that the treatment of the wound with Lp2621 gel exhibited considerable wound healing as compared to the vehicle and betadine-treated groups of mice, and is indicated by a reduction in the wound area as well as percent contraction ($p = 0.033$) of the wound (**Figure 7** and **Figures 8A,B**). Histopathological examination of wound tissues on day 7 (**Figure 9**) showed an enhanced proliferation of fibroblasts, vascularization, re-epithelization, collagen deposition, and the granulation of tissue in betadine- and Lp2621 gel–treated groups as compared to the vehicle-treated group. These results were corroborated by histopathological examination of the tissues, on day 14. The wound healing was incomplete in vehicle-treated mice, while in the betadine- and Lp2621 gel–treated groups of mice, the tissue was completely healed and appears to be histologically normal.

3.5.3 Efficacy of Lp2621 Gel on an Excision Wound Healing Model Infected With *S. aureus*

The efficacy of Lp2621 gel on the excision wound in the mice infected with *S. aureus* was evaluated. The results show that the treatment of infected wound with Lp2621 gel leads to a substantially quicker wound recovery compared to the vehicle and positive control as evident by a reduction in the wound area and an increase in the percent contraction ($p = 0.002$ and $p = 0.020$) of the wound (**Figure 10** and **Figures 11A,B**). The rate of wound healing activity was better in betadine- and Lp2621 gel–treated infected wounds than in the untreated infected wounds as observed on day 7 (**Figure 12**). The vehicle-treated infected wound tissues depict persistent inflammatory changes with the infiltration of inflammatory cells, mainly neutrophils, granulation of connective tissue in the wound area with numerous loops of blood vessels, fibroblast proliferation, and poor re-epithelization (**Figure 12A**). However, betadine- and Lp2621 gel–treated infected wound tissues depict re-epithelization of tissues with a reduced infiltration of leukocytes, increased fibroblastic activity, collagenation, and granulation of tissues (**Figures 12B,C**).

3.5.4 Cytokine Analysis

We further verified the role of Lp2621 in the immunoregulation of pro- and anti-inflammatory cytokines in the healing of normal and/or wounds infected with *S. aureus* infection. As shown in **Figures 13A–D**, the serum levels of pro-inflammatory cytokine

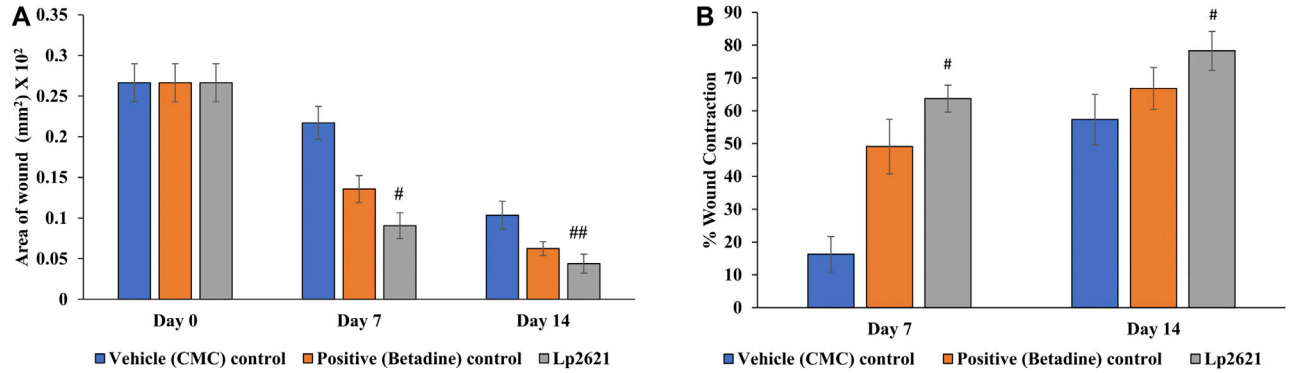

FIGURE 11 | Effect of topical treatment of Lp2621 gel on different groups: **(A)** on wound area infected with S. *aureus* and **(B)** percent wound contraction at different days post-wounding. Data are expressed as mean ± SD. $^{\#}$, and $^{\#\#}$ mean $p = 0.002$, and $p = 0.020$.

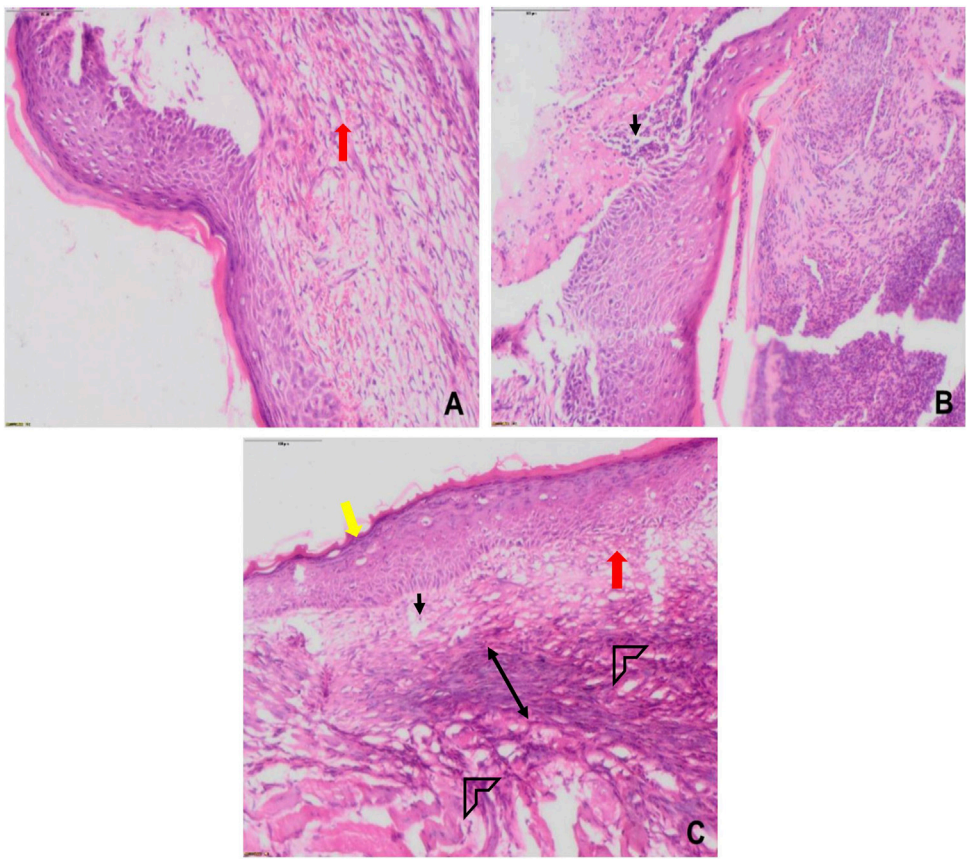

FIGURE 12 | Histological images of skin tissue from infected wounds on day 7: **(A)** vehicle (CMC), **(B)** Positive (betadine), **(C)** Lp2621 (scale bar = 100 μm). Fibroblasts (black), vascularization (red), re-epithelization (yellow), collagen deposition (arrow head), and granulation of tissue (double-sided arrow).

IL-6 were elevated in the initial phase of wound healing but declined on day 14. On the contrary, higher levels of IL-10 were observed during the later phase of wound recovery. However, this variation in levels of cytokines (IL-6 and IL-10) in normal and/or wounds infected with *S. aureus* infection was not statistically significant between the experimental groups on the respective day of the study.

4. DISCUSSION

In the present study, we assessed the antibacterial, hemolytic, antioxidant, and the wound healing properties of Lp2621 in A549 cells and excision wounds with and without *S. aureus* infection model in mice. Previous studies have reported the probiotic

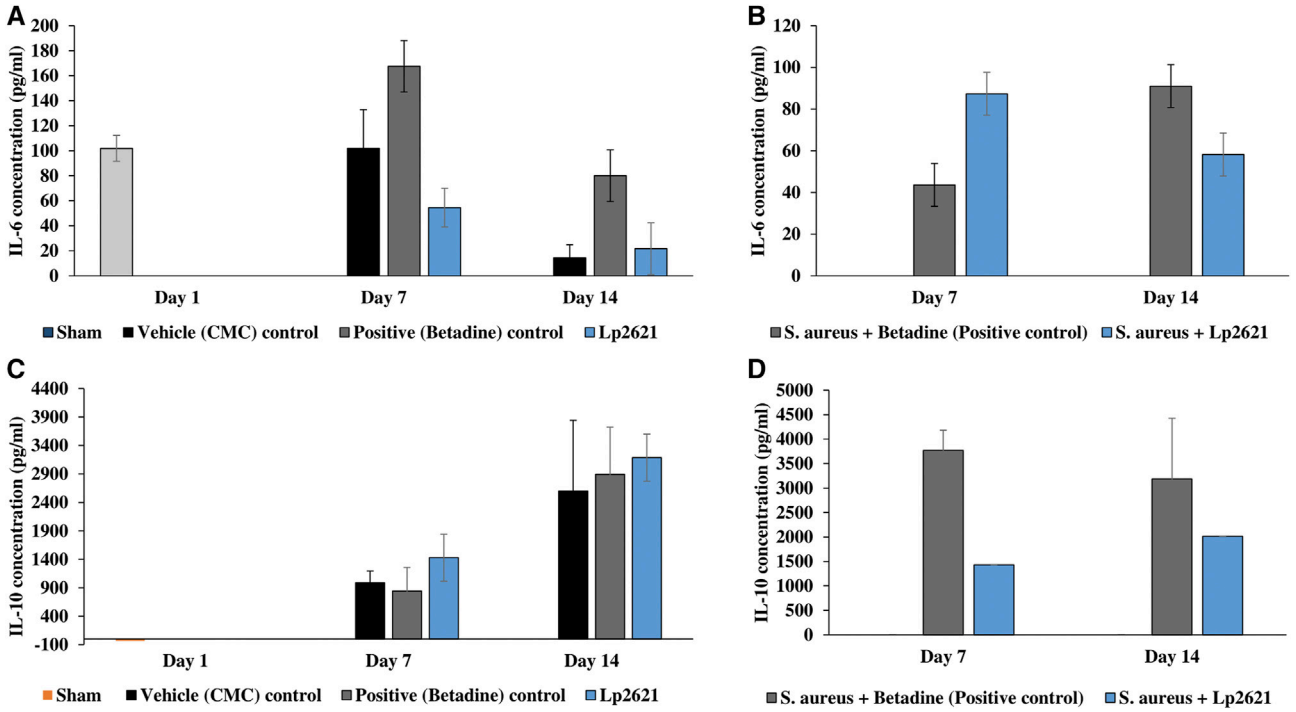

FIGURE 13 | Cytokine levels in the serum sample of mice. **(A,B)** IL-6 levels in the excision wound and the wound infected with *S. aureus*. **(C,D)** IL-10 levels in the excision wound and the wound infected with *S. aureus*. Data are representative of two independent experiments performed in duplicates and expressed as mean ± SE.

potential (Huang et al., 2013; Pop et al., 2016; Khalil et al., 2018; Monteiro et al., 2019) and antimicrobial activity of various *Lactobacillus* species (Halder et al., 2017; Prabhurajeshwar and Chandrakanth 2019; Qian et al., 2020) as well as *Lpb. plantarum* MTCC 2621 (Sreevani and Kumari 2013). The mechanisms behind the antibacterial activity of *Lactobacillus* strains are likely due to the production of antimicrobial compounds, resistance and competition for nutrients with other pathogenic bacteria, reduction of bacterial attachment to the mucosa, and modulation of the host immune system (Maria Tufail 2011; Giani et al., 2019).

The cutaneous wound healing activity of various *Lactobacillus* species has been previously reported in animal studies (Tsiouris and Tsiouri 2017). Another group demonstrated that the topical application of live *L. reuteri* DSM 17938 and its lysate induced anti-inflammatory activity by reducing the levels of pro-inflammatory cytokines (IL-6 and IL-8) (Khmaladze et al., 2019). The antimicrobial and *in vivo* wound healing potential of the probiotic VITSAMJ1 in rats has been studied previously (Sinha et al., 2019). Similar outcomes were observed in the burn wounds, where topical application of *Lpb. plantarum* could promote the wound healing (Satish et al., 2017). Probiotics such as *Lacticaseibacillus paracasei* and *Lpb. plantarum* significantly enhanced the production of IL-6 in the presence of IL-1β, an inflammatory cytokine in enterocytes (Caco2 cells), intermediated through hsp70 and hsp27 heat shock proteins (Reilly et al., 2007). Our findings (**Figures 7–12**) are consistent with the recent work by Khodaii and coworkers, where the wound healing activity was considerably promoted by the administration of *L. reuteri* extract by day 15 post-wounding (Khodaii et al., 2019). *L. reuteri* promoted wound healing *via* the PI3K/AKT/β-catenin/TGFβ1 pathway (Han et al., 2019). In another study, *Limosilactobacillus fermentum* enhanced the wound healing by promoting the production of anti-inflammatory and anti-pathogenic factors (Brandi et al., 2020). Ashoori et al. (2020) observed that the rate of wound healing was faster in the groups treated with both *L. reuteri* and *L. fermentum* supernatant-loaded chitosan nanogel (Ashoori et al., 2020). The metabolites of probiotics increased proteoglycan deposition, angiogenesis, reduced inflammation, and stimulated different growth factors (Matsumoto et al., 2005; Sonal Sekhar et al., 2014).

The histopathological examination of wound tissues in the present study (**Figure 9** and **Figure 12**) revealed angiogenesis and the recruitment of PMNL at the site of injury. These results are consistent with the earlier findings where a subcutaneous injection of LS into the mouse caused a continuous influx of polymorphonuclear leukocytes (PMNL) and macrophages in the wound area, and stimulated the inflammatory phase of the tissue repair (Halper et al., 2003). Histological changes were characterized by the infiltration of polynuclear neutrophils and dilatation of blood vessels along with a significant decrease in serum levels of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, IL-17, and IL-22, while an increase in the levels of IL-10 was observed in *Ligilactobacillus salivarius* LA307–treated mice (Holowacz et al., 2018). *Lactobacillus bulgaricus* and *Lpb. plantarum* accelerated wound healing by decreasing IL1β and TNFα, and upregulating IL-10 expression in diabetic Wistar rats (Mohtashami et al., 2020).

The probiotic strains have been consistently reported to modulate the pro-inflammatory cytokine, IL-6, and upregulate the level of anti-inflammatory cytokine, IL-10 (Karamese et al., 2016; Holowacz et al., 2018; Johnson et al., 2020). The favorable histological changes observed upon the treatment of the wound area with probiotic strains/extracts such as infiltration of polynuclear neutrophils and dilation of blood vessels are concomitant to the dynamic levels observed of IL-6 and is in accordance with the reported modulatory role (Holowacz et al., 2018; Johnson et al., 2020). Also, increase in angiogenesis, tissue regeneration, matrix remodeling, and repair are corroborated to increase in IL-10 and as such help in the regenerative process (Steen et al., 2020).

The findings of our wound healing study provide evidence that the topical application of Lp2621 to infected and uninfected wounds demonstrated rapid healing *via* enhanced angiogenesis, proliferation of fibroblasts, re-epithelization, and recruitment of PMNL. Another key finding of our study is that IL-6 level was elevated in the initial phase of wound healing followed by a decline by day 14. On the contrary, higher level of IL-10 was observed during the later phase of wound healing. The findings thus underscore the importance of cell-free supernatant of probiotic bacteria, *Lpb. plantarum* 2621 in treating both normal and *S. aureus*–infected wounds. These findings, therefore, suggest that probiotics and/or their metabolites have potential for the treatment of drug-resistant bacteria. Future research will be directed toward the development of probiotics/consortia of probiotics and their metabolites as alternatives to antibiotics for the effective treatment of drug-resistant bacteria, thereby thwarting the serious global threat of antimicrobial resistance.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of CSIR-Institute of Microbial Technology (IAEC approval number IAEC/20/18).

AUTHOR CONTRIBUTIONS

NK conceived and designed the experiments, funding acquisition, and project administration; AD, MP, PP, and SS performed the experiments; AP helped in microbiological experiments; SR and NK analyzed histopathological data; AD, MP, PP, SS, SR, and NK analyzed the results; and AD, SR, and NK wrote/reviewed and edited the paper. All authors reviewed and approved the article.

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REVIEW
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The Microbiota in Systemic Lupus Erythematosus: An Update on the Potential Function of Probiotics

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Systemic lupus erythematosus (SLE) is a kind of chronic diffuse connective tissue illness characterized by multisystem and multiorgan involvement, repeated recurrence and remission, and the presence of a large pool of autoantibodies in the body. Although the exact cause of SLE is not thoroughly revealed, accumulating evidence has manifested that intake of probiotics alters the composition of the gut microbiome, regulating the immunomodulatory and inflammatory response, which may be linked to the disease pathogenesis. Particularly, documented experiments demonstrated that SLE patients have remarkable changes in gut microbiota compared to healthy controls, indicating that the alteration of microbiota may be implicated in different phases of SLE. In this review, the alteration of microbiota in the development of SLE is summarized, and the mechanism of intestinal microbiota on the progression of immune and inflammatory responses in SLE is also discussed. Due to limited reports on the effects of probiotics supplementation in SLE patients, we emphasize advancements made in the last few years on the function and mechanisms of probiotics in the development of SLE animal models. Besides, we follow through literature to survey whether probiotics supplements can be an adjuvant therapy for comprehensive treatment of SLE. Research has indicated that intake of probiotics alters the composition of the gut microbiome, contributing to prevent the progression of SLE. Adjustment of the gut microbiome through probiotics supplementation seems to alleviate SLE symptoms and their cardiovascular and renal complications in animal models, marking this treatment as a potentially novel approach.

Keywords: autoimmunity, microbiota, probiotics, systemic lupus erythematosus, inflammation

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease, where a large pool of autoantibodies are produced, causing the immune system to attack its tissues, resulting in damage to multiple organs and systems throughout the body (Mu et al., 2015; Yacoub et al., 2018). Its main clinical features are multiple systems and organs involvement, repeated relapse and remission, and the development of a large pool of autoantibodies against double-stranded (ds) DNA (Lisnevskaia et al., 2014; Durcan et al., 2019; Fava and Petri, 2019). Individuals affected by SLE have extensive symptoms and course of the disease, the most frequent of which are fever, fatigue, facial butterfly erythema, photosensitivity, muscle or joint pain, arthritis, and renal symptoms (Goldblatt and

O'Neill, 2013). Moreover, patients with SLE have an increased incidence of atherosclerosis, thrombosis, arteritis, embolization, and vascular spasm (Kasselman et al., 2018). The most lethal outcomes in SLE patients are infection, severe multiple organ injury, especially damage to the nervous system and kidney (Lee et al., 2016; Yen et al., 2017). Production of the immune response in SLE is distinguished by an overreaction of B cell and T cell responses, and impaired self-tolerance to autoantigens. (Lisnevskaia et al., 2014; Durcan et al., 2019; Fava and Petri, 2019; Kiriakidou and Ching, 2020). The prevalence of SLE varies widely from region to region, with the current global prevalence approaching or even above 50 to 241 per 100,000 adults, among which the prevalence rates of African Americans, American Indians, and Alaska Natives are higher (Helmick et al., 2008; Ferucci et al., 2014; Somers et al., 2014; Rees et al., 2017; Nikolopoulos et al., 2020). The prevalence of SLE is higher among African Americans and Europe, which is less prevalent in Africa (Symmons, 1995; Pons-Estel et al., 2010). The severity of the disease may also vary by ethnic background, with patients of African and Latin American descent commonly more severe (Carter et al., 2016). Particularly, SLE is strikingly dominated by women of childbearing age, with nearly 10 female patients for every male suffering from the disease (Carter et al., 2016; Durcan et al., 2019; Fava and Petri, 2019; Fanouriakis et al., 2021). Among women aged 15 to 44, the ratio of women to men is 13:1 while the ratio is only 2:1 between children and the elderly (Petri, 2002; Danchenko et al., 2006).

A variety of medications are applied to SLE therapy, including glucocorticoids (GCs), antimalarial agents, nonsteroidal anti-inflammatory drugs (NSAIDs), immunosuppressive agents, and B cell–targeting biologics. NSAIDs block the synthesis of prostaglandins by attenuating the enzymes cyclooxygenases (COX-1 and COX-2) to counter inflammation and pain. The adverse reactions with the highest incidence of this drug are gastrointestinal gastritis, nephrotoxicity, fluid retention (Kiriakidou and Ching, 2020). Hydroxychloroquine is the cornerstone of lupus treatment (Kiriakidou and Ching, 2020). Hydroxychloroquine or other antimalarial agents have the effect of immunomodulatory and antithrombotic (Chrisman et al., 1976; Espinola et al., 2002; Wang et al., 2019). But the drugs can induce retinopathy, skin pigmentation, and rare cases of neuromuscular or cardiac toxicity (Marmor et al., 2016). GCs are the most commonly used agents in SLE-induced remission therapy and are consistently recommended by the guidelines as first-line agents for the control of SLE. (Gordon et al., 2018; Fanouriakis et al., 2019; Kiriakidou and Ching, 2020). The side effects that may occur after taking GCs are gastrointestinal adverse reaction, metabolic disorders, infections, weight gain, hypertension, psychiatric disorders, lipodystrophy, fractures, and adrenal suppression, which are mainly dose and time-dependent (Saag et al., 1994; van Staa et al., 2002; Wei et al., 2004; Da Silva et al., 2006; Warrington and Bostwick, 2006; Dixon et al., 2011; Sarnes et al., 2011). Furthermore, immunosuppressant therapy is suggested for SLE patients who continue to respond poorly to GCs and hydroxychloroquine combination therapy, or who cannot adjust the dose of GCs to a relatively safe dose. Immunosuppressive agents such as methotrexate inhibit DNA synthesis and increase the release of adenosine, but some patients were forced to stop using the drug because of intolerance to adverse reactions (Kiriakidou and Ching, 2020). What occurs most after the application of methotrexate are gastrointestinal side effects (nausea, vomiting, diarrhea), hepatotoxicity, and blood-related toxicity (anemia, leucopenia) (Sakthiswary and Suresh, 2014; Andreoli et al., 2017). For patients with refractory (ineffective after conventional treatment) or recurrent SLE, the administration of biological agents can reduce disease activity, disease recurrence rate and reduce hormone dosage (Wei et al., 2016; Alshaiki et al., 2018). Belimumab targets B-lymphocyte stimulator inhibits B-lymphocyte proliferation and activation (van Vollenhoven et al., 2012), but its common adverse effects are hypersensitivity reaction, gastrointestinal toxicity, myalgias, depression, migraine, infection (Lee and Song, 2018; Peterknecht et al., 2018). Rituximab depletes CD20-expressing B lymphocytes, but patients may occur an infusion reaction, infection, progressive multifocal leukoencephalopathy (rare) (Alshaiki et al., 2018; Peterknecht et al., 2018). In addition, other measures can be used to treat SLE. When SLE develops to severe or refractory levels, the addition of plasma exchange or DNA immunosorbent as adjunct therapy may be considered, which may ameliorate clinical symptoms rapidly but cannot improve the outcome (Kronbichler et al., 2016). In summary, all the medicines used in the treatment of SLE induce adverse reactions, whereas, probiotics supplementation appears to have no significant side effects clinically. Hence, it is necessary to further investigate probiotics for the exploration of theoretical basis as adjuvant therapy in SLE patients.

The precise pathogenesis of SLE is not entirely revealed, it is believed to be caused by the human immune system attacking self-tissues after being abnormally activated. The pathogenesis of SLE may be related to genetic, hormones and, environmental factors (infection, drugs, UVA light) (De Luca and Shoenfeld, 2019). Nevertheless, with research on intestinal flora dysregulation going depth recently, dysbiosis as a vital internal environmental factor has also been shown to be concerned with SLE (Meng et al., 2019). In 1994, Apperloo-Renkema et al. first demonstrated experimentally that alterations in intestinal microbiota composition can cause SLE in animal models, possibly due to a weakened defense of native gut microbes against foreign bacteria (Apperloo-Renkema et al., 1994). Repeated antigen stimulation may lead to changes in intestinal microecology, confusion of the immune system, and the body subsequently attacks its tissue by producing antibodies or sensitized lymphocytes (Zhang and Reichlin, 2008). The production of these antibodies is exacerbated by extensive inflammatory responses, which leads to a range of clinical symptoms and further complications associated with SLE (Zhang and Reichlin, 2008). Long-term use of probiotics is believed to neutralize an imbalance in the gut microbiota that results in the reduced antibody production and suppressed inflammatory response, leading to attenuation of severity, signs and, manifestation of SLE (Esmaeili et al., 2017). In a study assessing the resistance of intestinal microbiota to pathogen colonization in SLE patients and healthy controls,

the colonization resistance of patients with active SLE is lower than that of healthy people, which indicates that the incomplete normal intestinal microbiota may lead to more intestinal transfection of pathogenic bacteria (Apperloo-Renkema et al., 1994). The study presented above confirmed that the balance of intestinal microbiota is associated with the pathogenesis of SLE, but it is still unclear whether administering probiotics to restore normal intestinal flora and reduce inflammation may have therapeutic benefits for SLE patients or not (van der Meulen et al., 2016).

In brief, raising an understanding of how to ameliorate gut dysbiosis could help explore an alternative approach to prevent or alleviate SLE. Therefore, the alteration of microbiota associated with SLE was reviewed and the function and mechanisms of probiotics in the development of SLE animal models were also discussed.

AN INFLAMMATORY PATHWAY OF SLE

SLE is a multifactorial caused disease, and the pathogenesis is considered to be related to hormonal, environmental and genetic factors that lead to an intolerance to autoantigens (Rahman and Isenberg, 2008; De Luca and Shoenfeld, 2019). SLE is featured by the generation of autoantibodies, aggregation of autoreactive and inflammatory T cells, and abnormal production of inflammatory cells and pro-inflammatory cytokines (Buckner, 2010; Tsokos, 2011; Rastin et al., 2013). Autoantibodies produced by autoimmune B cells bring about the generation and accumulation of immunocomplex which do harm to multiple organs, containing the skin, joints, heart, kidneys, and brain (Zhang and Reichlin, 2008; Tsokos, 2011). Although SLE is regarded as mainly B cells mediated disease, there is some evidence indicating the significance of unbalanced regulatory T (Treg) cells in the development of SLE (Buckner, 2010; Ma et al., 2010; Talaat et al., 2015). In addition, it has been proven that improved T helper cell 17 (Th17) amount and effect play a crucial role by secreting pro-inflammatory cytokines, such as interleukin (IL)-17 and IL-23), as the primary trigger of autoimmune response, and these cytokines are related to the inflammatory formation and tissue damage in SLE (Crispín et al., 2008; Doreau et al., 2009; Chen et al., 2010; Pan et al., 2013). Studies have demonstrated that strengthening Treg cells restrain abnormal reactions of effector T cells, which can steadfastly alleviate autoimmune and inflammatory responses (Shevach, 2009; Lavi Arab et al., 2015; Reihani et al., 2015). It has been identified that patients with SLE have decreased numbers and functional deficiencies of Tregs as well as the resistance of effector T cells to the inhibitory effects of Tregs, which exert significant effects in the pathogenesis of SLE (Lyssuk et al., 2007; Valencia et al., 2007; Gómez et al., 2009; Esmaeili et al., 2017). It is reported that anti-inflammatory cytokines, such as transforming growth factor β (TGF-β) and pro-inflammatory cytokines, including IL-6, IFN-γ, and IL-23/IL-17 are drastic in every developmental stage of SLE (Su et al., 2012). Therefore, restoration of unbalanced cytokines and defective immune cells may be a potential remedial strategy for alleviating SLE manifestations (Esmaeili et al., 2017).

THE MICROBIOME AND PROBIOTICS

The Gut Microbiota

The intestine contains the largest complex mic-ecosystem in humans, which can be regarded as an independent organ in the body (Van de Wiele et al., 2016). According to high-throughput culture-independent sequencing analysis, the microbiome of the gut tract is more complicated than those of other parts of the body, with over 1,000 microorganisms identified so far, and the total biomass is close to 1,000 colony-forming units 10^{13}~10^{14}(CUF) (Claesson et al., 2009; Sankar et al., 2015). The intestine microbiota is dominated mainly by two phyla (approximately 90%) *Firmicutes* and *Bacteroidetes*, and the rest is involved in *Actinobacteria*, *Proteobacteria*, *Synergistetes*, *Verrucomicrobia*, *Fusobacteria*, and so on (Eckburg et al., 2005; de la Visitación et al., 2019). The existing methanogenic archaea, yeasts, and viruses (mainly phages) increase the complexity of the gut microbiota (Lozupone et al., 2012). Although only two phyla have predominance in the gut microbiota, there are striking differences in the intestinal microecology between people and people across different life cycles (Van de Wiele et al., 2016). Individual diversity in host genes, mode of delivery and lactation, geographic origin, age, diet, disease, drug uptake, and lifestyle contribute to differences in intestine microbiota composition (Ley, 2015). With the development of the functional characteristics of individual microbiota, growing evidence shows that the gut microbiota participated in critical activities related to disease and health. It has been proven that the functions of the human gut bacteria are to affect digestion, provide nutrients, form intestinal barriers and produce colonization resistance, regulate the development of intestinal epithelium, as well as to modulate the activation and progression of the immune system (Van de Wiele et al., 2016).

Therefore, any factors that disrupt the host-microbial balance (such as acute alters in dietary behavior; malnutrition; pathogen infection; inflammation; administration of anti-biological drugs; gastrointestinal surgery; etc.) may influence the homeostasis of microbiota which exerts a significant impact on the regulation of host immune functions (Ogura et al., 2003; Cho, 2008; De Filippo et al., 2010; Delzenne et al., 2011).

Probiotics

Probiotics are living organisms that regulate the gut microbiome in various ways to improve intestinal health. Probiotics can affect immune homeostasis by keeping a healthy microbial balance, and can also adjust mucus secretion through intestinal epithelial cells, thereby contributing to maintaining the stability of the mucus barrier and providing resistance to pathogen colonization (Bron et al., 2017; de Oliveira et al., 2017). Besides, Probiotics can promote the generation of multiple nutrients such as SCFAs and vitamins, which contribute to form the entire host intestinal microbiome (Yadav et al., 2013; de Oliveira et al., 2017). Moreover, probiotics participate in the degradation of toxic compounds and the production of antimicrobial compounds, like bacteriocins (de Oliveira et al., 2017). Therefore, these

probiotics can be used as a treatment option for immune-related diseases (Balakrishnan and Taneja, 2018).

Probiotics have been widely evaluated for their benefits in preventing or treating extensive diseases, including infection, inflammation, cancer, and autoimmune diseases in animal and human trails (Borchers et al., 2009). The recorded probiotic-inducing impacts include suppression of infection, immune regulation, prolonged remission of patients with ulcerative colitis, treating or preventing infective or antibiotic-associated diarrhea in both adults and infants, assisting in the eradication of Helicobacter pylori, improving nonalcoholic fatty liver disease and metabolic diseases, reducing the recurrence rate of colorectal cancer and alleviating lactose intolerance symptoms (de Vrese et al., 2001; Van Niel et al., 2002; Bengmark, 2003; Gill, 2003; Gionchetti et al., 2003; Kalliomäki et al., 2003; Tamboli et al., 2003). Probiotics have been reported to exert their beneficial effects mainly in three ways, containing competitive exclusion, antibacterial action, and regulation of immune responses. It has been found that the administration of immunoregulatory probiotics in the prevention or treatment of autoimmune diseases is mainly attributable to improving the inflammatory responses and modulating tolerance in the host to pathogens (Esmaeili et al., 2017).

Mechanism of Action of Probiotics

It has been discovered that probiotics influence each segment of the intestine, containing the intraluminal microbiota, the epithelial microbial, mucosal barrier, the lamina propria rich in lymphocytes and plasma cells, the blood vessels and nerves of lamina propria components, the underlying smooth muscles commanding movement and the mesenteric lymph nodes associated with systemic immunity (Liu et al., 2018). In mechanism, immunomodulatory probiotics are known to prevent inflammation and modulate immunity to improve SLE symptoms (Liu et al., 2018). As Liu et al. (Liu et al., 2018) summarized that short-chain fatty acids (SCFAs) generated by *bifidobacterium*, *lactobacillus*, and symbiotic bacteria combine and activate receptors (FFAR2, FFAR3, or GPR109a) on enterocytes to inhibit inflammatory responses by blocking nuclear factor-κ-light chain enhancer of B cells activation pathway. SCFAs also suppress histone deacetylases to facilitate amassing of Tregs and discharge glucagon-like protein-1/peptide tyrosine to respond to the enteric and central nervous system, thereby affecting intestinal homeostasis and motion. They also induce tolerogenic dendritic cells (DCs), which induce immature $CD4^+$ T cells to differentiate into Tregs. The above response restraint the generation of cytokines via neutrophils and macrophages by binding to receptors. Adenosine and its derivative inosine interact with adenosine receptor-2A expressed on T cells to enhance Treg effects and suppress TH1 and TH17 subsets inflammation. Histamine generated by *L. reuteri 6475* reacts on H2 receptor located in intestinal epithelial cells and macrophages to decrease the secretion of proinflammatory cytokines, containing tumor necrosis factor (TNF)-α, MCP (monocyte chemoattractant protein)-1, and IL-12. In conclusion, the pivotal metabolites generated by probiotics exhibit anti-inflammatory properties and improvement of the intestinal barrier function during the disease.

THE MICROBIOTA STUDIES IN SLE

Studies that described the microbiota of SLE are relatively limited, although the increasing prevalence of Crohn's disease (CD) in patients with SLE (Shor et al., 2016) has sparked interest in its involvement. Although the pathogenesis of SLE is not completely understood, an imbalance in the microbiome has been manifested to be associated with the establishment of SLE (Hevia et al., 2014; De Luca and Shoenfeld, 2019). Until now, human studies that investigate the connection between the microbiome and SLE initiation are observational case-control studies that compare differences of the human microbiome in areas like the gut or buccal cavity between SLE patients and controls (Arron et al., 2014; Hevia et al., 2014; Zhang et al., 2015; López et al., 2016). Therefore, revealing the microbial composition and possible function of these microbes in SLE patients may illuminate the cause and development, and may even find diagnostic biomarkers.

SLE patients have compositional and functional alterations in gut microbiota, possibly due to a weakened defense of native gut microbes against foreign bacteria (Apperloo-Renkema et al., 1994). Noticeable increase of several genera, including *Rhodococcus*, *Klebsiella*, *Eggerthella*, *Prevotella*, *Eubacterium*, and *Flavonifractor*, has been found in patients with SLE, whereas, *Dialister* and *Pseudobutyrivibrio* decreased (Hevia et al., 2014; He et al., 2016; Chen et al., 2017). Quantitative polymerase chain reaction analysis confirmed that the ratio of *Firmicutes* to *Bacteroidetes* was lower in patients with SLE, and the abundance of some families of *Firmicutes* was decreased (López et al., 2016) (Hevia et al., 2014; Neuman and Koren, 2017; van der Meulen et al., 2019) (**Table 1**). Such alterations are also present in other diseases, such as Crohn's disease and type 2 diabetes mellitus (Man et al., 2011), suggesting that an overall imbalanced microbiota state is not specific to SLE (Larsen et al., 2010). A similar study on the composition of gut microbiota in 45 Chinese patients with SLE was in accordance with the results mentioned above, showing lower *Firmicutes* and higher *Bacteroidetes* in SLE patients (He et al., 2016). Downregulating inflammation can be achieved in several ways, such as elimination of apoptotic cells and cell debris, clearance of oxidized lipids, and blocking the stimulation of mitogen-activated protein kinase (MAPK) and other pro-inflammatory cytokines (Grönwall et al., 2012; López et al., 2016). As the anti-dsDNA titer increased, the frequency of the Synergistetes, which was positively associated with the rate of *Firmicutes* to *Bacteroidetes* in healthy controls, verged to decrease in SLE patients and was present a significantly negative association with the level of proinflammatory cytokines IL-6 in serum, meanwhile, correlating positively with natural protective IgM anti-phosphorylcholine secreted by B1 cells (López et al., 2016).

In female SLE patients, studies have shown the abundances of *Lactobacillaceae* decreases, while the levels of *Lachnospiraceae* increase, both of which belong to the *Firmicutes phylum* (Zhang

TABLE 1 | The shift of microbiota in SLE.

Bacteria	Changes	Mechanism of pathogenesis/potential metabolic function/other associations	Ref
Bifidobacterium bifidum	↑	• prevents excessive activation of CD4(+) lymphocyte • generates fewer $CD25^{high}$ cells • generates IL-17, Th17, and functional Treg • reduces IFNc and TNFa • reverts the up regulatory effect on CD25 expression • reduces the percentage of Foxp3+ cells included within the $CD25^{high}$ population • induces phenotypic DC maturation	López et al. (2011); López et al. (2016)
*Clostridia strains**	↑	• restores Th17/Th1 balance • reduces the IL-17/IFNγ ratio induced by DCs • reduces the IL-17/IFNγ balance	López et al. (2016)
F/B ratio	↓	• increases inflammation • overrepresents oxidative phosphorylation and glycan utilization pathways • some effects are species-dependent	Hevia et al. (2014); Neuman and Koren (2017); van der Meulen et al. (2019)
Bacteroidetes	↑	• glycan-degrading activity • associated mucin-degrading sulfatase activity contributing to impaired epithelial cell layer and chronic inflammation	Hevia et al. (2014)
Firmicutes *Prevotella spp.*	↓ ↑	• produces butyrate • considered a gut commensal associated with a fiber-rich diet • SCFA producer	Hevia et al. (2014) He et al. (2016); Ley (2016); Chen et al. (2017)
Eggerthella (Coriobacteriaceae)	↑	• Not well characterized yet • believed to be an emerging pathogen	Gardiner et al. (2015); He et al. (2016)

Abbreviations: F/B, Firmicutes/Bacteroidetes*; TH, T helper; DC, Dendritic Cells; IL, interleukin; IFN, interferon; Foxp3+, forkhead Box P3+; ↓, decrease; ↑, increase; *, Two mixed strains of clostridium (*Ruminococcus obeum DSM25238 *and* Blautia coccoides DSM935*).*

et al., 2014; Mu et al., 2015; Neuman and Koren, 2017). Notably, comthe level of abutyrate-producing bacterium *Lachnospiraceae* was augmenter in SLE patients compared with healthy controls, thus *Lachnospiraceae* or any bacterium that produces butyrate may be unable to inhibit inflammation in SLE cases (Kakiyama et al., 2013; Zhang et al., 2014; Kasselman et al., 2018; Luo et al., 2018). Further, patients with SLE tend to generate more $CD25^{high}$ cells, whereas *Bifidobacterium bifidum (B. bifidum)* strain can revert to the up regulatory effect (López et al., 2016). On the other hand, the microbiota isolated from the feces of SLE patients has been found to accelerate the activation of lymphocytes and the differentiation of Th17 from primitive $CD4^+$ lymphocytes (López et al., 2016). Additionally, *B. bifidum* may prevent lymphocyte activation whereas mixtures of two Clostridia strains supplementation, including *Ruminococcus obeum DSM25238* and *Blautia coccoides DSM935*, restore Th17/Th1 balance (Atarashi et al., 2011; Atarashi et al., 2013; López et al., 2016). Alternatively, increased numbers of *Selenomonas*, *Veillonella*, *T. denticola*, and *Leptotrichia* are directly associated with raised concentrations of inflammatory factors like IL-6, IL-17, and IL-33, which are indicative of a decline in oral microbial species diversity in SLE patients (Corrêa et al., 2017).

ROLES OF PROBIOTICS AGAINST SLE

As a result of the above-mentioned findings, researchers believe that SLE treatment with probiotics (**Table 2**), has already presented some benefits like in other autoimmune diseases, can help ameliorate the symptomatology of disease (Schiffer et al., 2011; Zamani et al., 2016). Studies in animal and human trials have identified the potential benefits of probiotics in the alleviation and suppression of inflammation and autoimmune responses (Liu et al., 2018). Research in the SLE animal model has demonstrated that certain probiotic strains, including *B. bifidum*, *Lactobacillus*, *Ruminococcus obeum*, and *Blautia coccoides*, contribute to regulating excessive inflammation and restore tolerances (Esmaeili et al., 2017).

Researchers found that enteral administration of combinations of *lactobacilli* or *L. reuteri* alone in MRL/LPR mice, SLE mouse models, can skew the balance of Treg–Th17 toward Treg cell advantage in the kidneys, reduce endotoxemia, decrease the concentrations of dsDNA-reactive IgG, decrease urinary protein, and ameliorate the survival rate of patients (Mu et al., 2017). These outcomes were related to a shift in intestinal microbiota and extension of *Lactobacilli*, *Clostridiales*, and *Desulfovibrionales*. Mu et al. (2017) found that *Lactobacillus spp.* supplementation plays an anti-inflammatory role through reducing IL-6 and enhancing IL-10 generation in the intestine. The supplement of therapeutic *Lactobacillus* enhanced circulating IL-10 and declined IgG2a, which is regarded as a main immune deposition in MRL/lpr mice kidney. These benefits were observed in female and unsexed male mice, rather than in male functional mice, indicating that intestinal flora may regulate inflammation in a sex hormone-dependent pattern. According to Mardani et al.(2018), the consumption of *Lactobacillus*

TABLE 2 | Effects of probiotics in SLE animal models.

Probiotic strain	Model	Effects	Ref.
*Lactobacillus strains**	MRL/lpr, mice	• suppresses the generation of IL-6 IL-10 • decreases the production and renal deposition of pathogenic IgG2a • suppresses pathogenic Th 17 cells • increase Treg cells • rebalances T cell subsets in the kidney	Mu et al. (2017)
Lactobacillus	Female pristane-induced BALB/c mice	• decreases IL-6 • reduces Th1–Th17 polarization • reduces the number of Th17 cells • decreases the expression of IL-17 mRNA and IL-17 protein levels • decreases the level of anti-dsDNA, ANA, anti-RAP • enhances Tregs and the expression level of FoxP3	Mardani et al. (2018); Khorasani et al., 2019)
Lactobacillus reuteri GMNL 263	NZB/W F1	• decreases TLR-4, TLR-5, TLR-7, and TLR-9 • declines the generation of IL-1β, IL-6, and TNF-α • increases the differentiation of $CD4^+CD25$ + FoxP3+ T cells • increases the proportion of $CD4^+CD25^+$ T cells in CD4+T cells • increases the mRNA level of Foxp3 in $CD4^+CD25$ + T cells	Hsu et al. (2017); Tzang et al. (2017)
Lactobacillus paracasei GMNL 32	NZB/W F1	• reduces the expression of IL-1β, IL-6, and TNF-α • down-regulates TLR-4, TLR-5, TLR-7, and TLR-9 • increases heart weight and the ventricular wall thickness	Hsu et al. (2017); Hu et al. (2017); Tzang et al. (2017)
Heat-Killed Lactobacillus reuteri GMNL-263	NZB/W F1	• reduces TUNEL-positive cells, Fas death receptor-related elements, and TNF-R1 • increases the levels of survival protein phospho-AKT • decreases MMP-9 and the levels of MMP-9 proteins	Yeh et al. (2021)
Lactobacillus fermentum CECT5716	NZB/W F1	• reduces the elevated T, B, Treg, and Th-1 cells in mesenteric lymph nodes • decreases the plasma levels of IL-17a, IL-10, IFN-g, TNF-a, and IL-2 to normal levels • decreases gene expression of IL-6, IL-β, THF-α, and TLR-4 in the aorta	Toral et al. (2019); de la Visitación et al. (2020)
Bacteroides fragilis ATCC 25285	Female C57BL/6J and B6.MRL-Faslpr/J lupus-prone mice	• reduces the production of TNF-α, IL-6, and MCP-1 • promotes CD1d production in B cells by Est-1 pathway • inhibits CD86 expression to repair the immune response of B cells • decreases the level of anti-dsDNA, total IgM, total IgG, BUN, Cre, and RBP in serum • increases CD1d expression level	Li et al. (2020)

*Abbreviations: IL, interleukin; Th, T-helper; Treg, regulatory T cells; Foxp3, forkhead Box P3; ANA; TNF, tumor necrosis factor; TLR, toll-like receptor; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase, 2′-deoxyuridine, 5′-triphosphate (dUTP)-mediated nick-end labeling; MMP-9, matrix metallopeptidase 9; MCP, monocyte chemoattractant protein-1; BUN, blood urea nitrogen; *, A mixture of 5 Lactobacillus strains (*L. oris, L. rhamnosus, L. reuteri, LactobaCallus johnsonii, *and* L. gasseri*).*

delbrueckii and *actobacillus rhamnosusin* to Female pristane-induced BALB/c mice improved the symptoms of SLE, exhibit anti-inflammatory properties by attenuating the generation of Th7 and down-regulating its major cytokines of IL-17a, one of the critical mediators in the formation and progression of inflammation.

L. reuteri GMNL 263 can down-regulate cytokine levels and repaired Tregs in NZB/W F1 mouse model, which is distinguished by oxidative stress and reduction of regulatory Tregs levels in circulation (Tzang et al., 2017; Liu et al., 2018). Alternatively, *L. GMNL 263(GMNL 263)* showed a diverse mechanism in the NZB/W F1mouse model of SLE (Tzang et al., 2017). These probiotics strains can enhance the production of Treg lymphocytes and the levels of transcription factor fork head box P3 (FoxP3), which is the hallmark of a natural Treg. These cells are in charge of regulating these pro-inflammatory lymphocytes and have significant anti-inflammatory characteristics. Besides, TLR-4, TLR-5, TLR-7,

and TLR-9, which are the common pathogen-associated molecular pattern receptors that mediate the inflammation progression in the liver, were decreased and the antioxidant activity was increased under probiotics treatment (Hsu et al., 2017; Tzang et al., 2017). In addition, *GMNL 263* also promoted the differentiation of CD4+CD25+FoxP3+ T cells and the proportion of CD4+CD25+ T cells number in CD4+T cells of spleen and enhanced the expression of Foxp3 mRNA in $CD4^{+}CD25+$ T cells (Hsu et al., 2017).

In similar trials, the above alterations of TLRs and oxidative stress were also found using probiotics *L. paracasei GMNL 32(GMNL-32)* and *L. reuteri GMNL 89*, although *GMNL 263* presented an effect on Treg expression in those cases (Hsu et al., 2017). The SLE-associated inflammation was also decreased with the administration of these probiotics, through enhancing the activity of antioxidation in serum and levels of $CD4^{+}CD25^{+}$ regulatory T cells in NZB/W F1 mice (Tzang et al., 2017). Moreover, in the treatment of these three probiotics strains, pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 declined in the liver by inhibiting nuclear factor kB (NF-κB) and the signaling pathway of mitogen-activated protein kinase (Hsu et al., 2017; Hu et al., 2017). Specifically, *GMNL-32* supplement attenuated left ventricular hypertrophy and the cardiac cell apoptosis in this genetic model of lupus (Hu et al., 2017; Tzang et al., 2017). These results indicated that oral supplement of several probiotic strains, such as *GMNL32*, *L. reuteri GMNL-89*, and *L. reuteri GMNL263*, to NZB/W F1 mice can not only mitigates hepatic inflammation and apoptosis caused by SLE, but also presents a protective function on cardiac cells of lupus-prone mice (Hsu et al., 2017; Hu et al., 2017; Tzang et al., 2017).

Yeh et al. (2021) first revealed the preventive effect of *Heat-killed L. reuteri GMNL-263* on expanded interstitial spaces and abnormal myocardial structures in the hearts of NZB/W F1 mice, and lowered area of fibrosis and rescues cardiomyocyte arrangement, which demonstrate the clinical applications of the *Lactobacillus* in SLE-related cardiovascular diseases therapy. Because *Heat-Killed L. reuteri GMNL263* prevented the development of the proinflammatory response, cardiac and renal hypertrophy complications in SLE were averted (Yeh et al., 2021). Compared with the controls, the anti-apoptotic effects were observed in the NZB/W F1 mice, and the significant declines of TUNEL-positive cells, Fas death receptor-related elements, and apoptosis were also detected after the consumption of *GMNL-263*. Additionally, administration of *L. reuteri GMNL-263* to NZB/W F1mice present markedly higher levels of phospho-AKT (survival protein) than in NZB/W F1 control group. In fact, feeding *L. Paracasei GMNL 32* was also detected to exhibit a similar protective pathway and prevent cardiac complications associated with SLE in NZB/W F1 mice. The Heat-killed *L. reuteri GMNL-263* was a kind of dead bacteria, which was killed after heat treatment at 121°C for 5 min in 0.9% sterile NaCl and were made into powder freeze-dried. During the experiment, the powder was dissolved into a probiotic solution and fed to mice. Live probiotics provide barrier protection and immune system modulation; while components of dead cells exert an anti-inflammatory response in the gastrointestinal tract. Both live and dead probiotics can exert specific actions. To sum up, both *L. reuteri GMNL-263* and *L. paracasei GMNL 32* have been observed to exert cardioprotective properties by reducing TNF-R1, Fas-associated protein with death domain (FADD) and fibrosis proteins matrix metallopeptidase 9 (MMP-9).

Research has found *L. fermentum CECT5716(LC40)* protects the kidney and cardiovascular complications as well as disease activity in a female mouse model of SLE (Toral et al., 2019; de la Visitación et al., 2020). The administration of the immune-modulatory bacterium LC40 could increase the number of Bifidobacterium in the intestine of female NZB/WF1 mice. *LC40* can reduce the activity of lupus and splenomegaly in SLE mice, improving the integrity of the intestinal barrier, reducing the plasma level of lipopolysaccharide (LPS), and subsequently decreasing the immune activation, which was characterized by reduced T and B cells in mesenteric lymph nodes (MLNs) and declined plasma pro-inflammatory factors, containing TNF-α, IFN-γ, IL-17a, and IL-21. Since probiotics prevented the progression of proinflammatory responses, complications related to SLE, such as cardiac and renal hyperplasia, were prevented (Toral et al., 2019). Another research reported that treatment with *LC40* decreased the enhanced plasma anti-dsDNA, endotoxemia, and hypertension in NZB/WF1 mice. Meanwhile, *LC40* also protected lupus mice from deterioration in renal function and kidney damage, as well as suppressing immune-complex deposition and inflammatory infiltration in glomerular, tubulointerstitial, and vascular lesions (de la Visitación et al., 2020).

In one recent experiment conducted by Li et al. (2020), oral supplementation of *Bacteroides fragilis (B. fragilis) ATCC 25285* reduced autoantibodies levels and symptoms of lupus nephritis in MRL/lpr mice. The results confirmed that *B. fragilis ATCC 25285* could improve the expression CD1d in B cells through Est-1 pathway, but suppress the expression of CD86 through SHP-2 signaling pathway to restore the immune response of B cells. Furthermore, levels of anti-dsDNA, total IgG and total IgM, as well levels of BUN, CRE, and RBP in serum decreased in MRL/lpr mice. In parallel, *B. fragilis ATCC 25285* was found to play a role in restoring the balance of Th17/Treg in MRL/lpr mice, as it does in other autoimmune diseases (Li et al., 2020).

Through the above research, we have an overall understanding of the beneficial role of probiotics in adjuvant therapy of SLE, particularly the regulatory function of Treg and Th17 (de la Visitación et al., 2019). Dendritic and T Treg cells, cytokines like IL-6, IFN-γ, IL-17, and IL-23 are currently regarded as the most dominant mediators of dysregulation in the tolerated condition (Esmaeili et al., 2017). Different strains of probiotics may exhibit different beneficial functions but still fall within the same species. In this regard, live versus heat-killed probiotics present different properties, such as *L. reuteri GMNL-263* and *Heat-killed L. reuteri GMNL-263* (Adams, 2010). Therefore, further exploration of the potential mechanisms of probiotics is necessary, which will not only contribute to the cause and progression of SLE but may also

support an alternative strategy for the comprehensive treatment of SLE, such as renal, cardiovascular, and hepatic complications.

DISCUSSION

It is well known that there is a connection between microbes and autoimmune diseases. Alterations of the microbiome, namely "dysbiosis" can cause autoimmune disease influenced by the factors of certain genetic backgrounds and environments (De Luca and Shoenfeld, 2019). Dysbiosis can occur with the following three situations: reduction of beneficial microorganisms, overgrowth of potentially harmful microorganisms, and decrease of microbial diversity (DeGruttola et al., 2016). In order to ameliorate the adverse effects produced by microbial imbalances during the course of the disease, it may be possible to reestablish a healthy microbiota by supplement multiple probiotic strains, such as *Bifidobacteriaum spp.*, *Lactobacillus spp.*, *Lactococcus spp.*, *Pediococcus spp.*, or more varieties (Solis et al., 2002; Homayouni et al., 2014). Furthermore, fecal microbiota transplantation (FMT) is the transplantation of healthy fecal fluids into the gut of the recipient to restore a stable intestinal flora, which affects both the endogenous and host microbes (Gough et al., 2011).

Theoretical risks of probiotics have been illustrated in case reports, including systemic infections, harmful metabolic activities, gene transfer, extreme immune activation in susceptible populations, and gastrointestinal adverse reactions (Doron and Snydman, 2015). Notably, the most frequently reported single event is fungemia caused by consumption of *Lactobacillus acidophilus* and *Lactobacillus casei* (Barton et al., 2001; De Groote et al., 2005; Ledoux et al., 2006; Vahabnezhad et al., 2013). Meanwhile, incidents of endocarditis caused by both *Lactobacillus* and *Streptococcus* probiotics have also been reported (Mackay et al., 1999; Doron and Snydman, 2015). Although probiotics supplements appear to have no clinically significant side effects, the administration of probiotics in susceptible individuals should be treated with caution.

In conclusion, the existing evidence manifests that some probiotics, such as *Lactobacillus*, which can restore dysbiosis and enhance intestinal barrier function may prevent the occurrence of cardiovascular and renal complications of SLE and alleviate its symptoms. The mechanism of intestinal microflora imbalance inducing the occurrence and development of SLE may be associated with the abnormal T cell subsets, particularly the abnormal levels of Naïve CD4+T, γδT, Tfh, Treg, and Th17 cells. Further exploration of the mechanism by which the probiotics influence the disease state of SLE, most likely through inflammation and the immune system, may contribute to the progression of future clinical treatments. Therefore, it is significant to shed light on the variation of intestine microbiota to exhibit anti-inflammatory properties, and potentially they can be considered as biomarkers to reflecting disease status. Particularly important is that more animal trials combined with clinical studies are needed to further elucidate the mechanisms for the effect of probiotics, meanwhile, to unravel whether specific probiotics bacteria have a positive impact on the treatment or prevention of SLE to develop novel therapeutic targets.

AUTHOR CONTRIBUTIONS

XG, XY, QL, and XS searched and reviewed literature. XG defined the structure of the paper and wrote the original draft. HZ and YY supervised the study and revised the paper. All authors reviewed and approved the final format.

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MINI REVIEW
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Probiotics and Vitamin D/Vitamin D Receptor Pathway Interaction: Potential Therapeutic Implications in Inflammatory Bowel Disease

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Inflammatory bowel diseases (IBD) are chronic conditions of unknown etiology and immunomediated pathogenesis. In the last years, the comprehension of the complex mechanisms involved in the intestinal mucosal homeostasis, and the analysis of the alterations potentially leading to inflammatory pathologic states, has consistently increased. Specifically, the extraordinary impulse in the field of research of the intestinal microbiome has opened the door to the investigation of possible novel approaches to the diagnosis, management and therapeutic applications in IBD. In line with that, administration of probiotic bacteria has been intensely evaluated, leading to much more exciting results in experimental models than in clinical practice. Considering the consistent heterogeneity of the available studies on probiotics, the increased knowledge of the properties of the single bacterial species would ideally lead to unravel potential mechanisms of action that may bring therapeutic applications in specific pathologic condition. Among the relevant molecular pathways for mucosal homeostasis maintenance, the vitamin D/vitamin D receptor (VDR) pathway has been intensely studied in the very last years. In fact, besides osteometabolic functions, the vitamin D exerts important homeostatic effects in the organism at multiple levels, such as immunomodulation, inflammation control, and microbiota regulation, which are likely to play a relevant role in intestinal mucosa protection. In the present review, recent findings about probiotic applications in IBD and mechanisms of action linking vitamin D/VDR pathway to IBD are reported. Available evidence for probiotic effect on vitamin D/VDR are reviewed and potential future application in IBD patients are discussed. At present, many aspects of IBD pathogenesis are still obscure, and current therapeutic options for IBD treatment are at best suboptimal. The increasing comprehension of the different pathways involved in IBD pathogenesis will lead to novel findings ideally leading to potential clinical applications. Microbiota manipulation and vitamin/VDR pathway appear a promising field for future research and therapeutic developments.

Keywords: inflammatory bowel disease, probiotics, vitamin D, vitamin D receptor, homoestasis

INTRODUCTION

In the last decades, virtually every field of human science has been involved and shaked by the effect of the so called "microbiome revolution." In fact, under the stimulation of novel culture-independent laboratory techniques, that allowed a thorough evaluation of bacterial intestinal species, and thank to an increased comprehension of the molecular mediators of microbiota-intestine interaction, an exponential and intensive interest rising has led to a consistent impulse to this field of research (Rescigno, 2017). Consequently, the idea that the complex eco-system hosted in our gut, collectively comprised in the term "microbiota," could represent a virtual organ of our organism, with a fundamental role in health maintenance, has more and more decisely taken pace (Marchesi et al., 2016). In line with that, therapeutic manipulation of the microbiota, by means of diet, nutraceuticals, antibiotics, pre- and probiotics has been proposed and investigated in many areas of medicine, with mixed results (Preidis and Versalovic, 2009).

Specifically, the idea of the utilization of beneficial bacterial species for health purposes has been proposed as early as in the beginning of 20th century with the pioneer studies by Elie Methnickov, but it's at the turn of the new Millennium that the scientific research in that field has consistently grown and expanded (Marchesi et al., 2016). The probiotics, defined by the World Health Organization (WHO) as living organisms with beneficial health effect whether ingested in adequate quantity, has been therefore intensely investigated in experimental models and clinical studies, with more striking results in the former setting comparing with in the latter, mostly due to the extreme dishomogeneity of literature data (Suez et al., 2019). At present, current research on probiotic bacteria is following two main lines. From one side, bacteria with a strong history of empirical utilization and safety data, mainly from Lactobacilli and Bifidobacteria genera, have been rigorously and carefully investigated in pre-clinical and clinical studies, in order to propose and solidly support clinical utilization in specific situations (Kleerebezem and Vaughan, 2009). On the other hand, by means of an accurate microbiota composition analysis, difference between health subjects and patients with different diseases has been characterized, with the final ideal goal to identify bacterial species of particular relevance for the pathologic condition, potentially useful as novel probiotic bacteria ("next generation probiotics") to supplement for therapeutic purposes (O'Toole et al., 2017). Regardless the research approach and notwithstanding the actual flaws for an evidence-based utilization of probiotics, the clearest concept emerged is that probiotics are not the same, but many molecular and therefore potential clinical effect are often species-specific and not generally extendable (McFarland et al., 2018). Accordingly, the generic term "probiotic" has nowadays lost its sense, considering that, at present, and many more in the future, the identification of specific molecular properties of well characterized bacterial species, and the correct and aimed positioning in a specific clinical setting, it's most probably the key to the implementation of probiotics utilization as a therapeutic option in medicine.

Among the infective and inflammatory pathologies where probiotics' application has been investigated, inflammatory bowel diseases (IBD) still represent one of the most promising and yet debated (Ghouri et al., 2014). IBD are a group of diseases, whose two main forms are represented by ulcerative colitis (UC) and Crohn's disease (CD), clinically characterized by intermittent/recurrent symptoms of active disease (abdominal pain, diarrhea, bloody stool) and remittent phases. Even though these two entities share pathogenetic similarities, they present peculiar morphological and clinical features. UC is characterized by a chronic inflammation of the superficial layer of the colonic mucosa, initiating in the rectum and with a variable proximal extension, while in CD the mucosal inflammation is transmural and may affect virtually every segment of the GI tract with skip lesions, and may be characterized by prevalence of inflammation or complications such as stenosis and fistulas (Abraham and Cho, 2009). Among available pharmacological treatments there are mesalamine, corticosteroids, antibiotics, immunosuppressant and biologic drugs, with the latter representing the mainstay of treatment for moderate-severe disease (Lamb et al., 2019). Despite conventional and immunomodulatory therapy, still many patients do not respond adequately, so that the research and the development of novel pathways involved in disease occurrence, to be targeted for therapeutic purposes, are largely needed. Among possible involved molecular pathways, in very recent years the vitamin D/vitamin D receptor (VDR) interaction has been consistently proposed (Kellermann et al., 2020). In fact, besides its well characterized role in bone metabolism, vitamin D has been recently highlighted as an important molecular mediator for intestinal homeostasis, due to important immunomodulatory and anti-inflammatory effect (Del Pinto et al., 2017). Since bi-univocal links between microbiota and vitamin D has been hypothesized, the idea of a potential therapeutic application of probiotic bacteria and vitamin D in IBD patients appears more than attractive.

In the present narrative review we intended to critically analyse pre-clinical and clinical available data on potential influence of probiotic and vitamin D pathway interaction in IBD patients. The concomitant use of probiotic and vitamin D could be helpful in IBD patients both for the single potential positive effect on intestinal inflammation that probiotics and vitamin D may exert singularly, and for a real molecular interaction with a reciprocal amplification of effect. Therefore, we briefly summarized the experimental and clinical data for probiotic and vitamin D efficacy in IBD separately, and then we explored the possible interaction at molecular level and the clinical effect of probiotic/vitamin D concomitant administration.

PROBIOTICS IN IBD: POTENTIAL MECHANISM OF ACTION AND CLINICAL EVIDENCE

Evidence for a microbial influence in IBD onset and/or development comes from initial observations from germ-free animals and in patients with fecal diversion, indicating a negative role of intestinal

bacteria (Rutgeerts et al., 1991; Taurog et al., 1994). More recent data suggest that an altered balance between protective and pathogenic bacteria occurs in IBD patients ("dysbiosis"), potentially contributing to the initiation and progression of a deregulated chronic inflammation (Caruso et al., 2020). Indeed, a consistent set of experimental and pre-clinical data indicate potential mechanisms of action by which specific probiotic bacteria may exert a beneficial effect on chronic intestinal inflammation (Ciorba, 2012). In fact, probiotics may contrast the dysbiosis by reducing pathogenic bacteria and stimulating beneficial ones, such as butirrate-producing bacteria (Markowiak-Kopec and Slizewska, 2020). Moreover, they may temporary colonize the intestinal mucosa and directly interact with specific receptors of the innate immune system, namely the pattern recognition receptors - PRR (i.e., nucleotide-binding oligomerization domain - NOD and toll-like receptors - TLRs), thus exerting an immunomodulatory effect (Bermudez-Brito et al., 2012). As a consequence, epithelial functions are enhanced, with stimulation of cytoprotective factors, improving of epithelial cells survival, stimulation of mucus and anti-bacteria molecules production, reduction of intestinal permeability (Ohland and Macnaughton, 2010). The increase of the intestinal barrier efficacy reduces the antigen load to the sub-mucosal compartment, and for that reason, and for a direct effect of probiotics on dendritic cells and lymphocytes, adaptive pro-inflammatory immune response is prevented and reduced, with a reduction of pro-inflammatory cytokines (i.e., TNF, IFN, IL-17) and a stimulation of regulative mediators (i.e., IL-10, TGFb, IL-4) (Pagnini et al., 2013). Unfortunately, the impressive experimental data have not be followed so far by convincing clinical results, and clinical trials in IBD patients have been characterized by a dramatic dishomogeneity in terms of probiotic used, doses and duration of the therapeutic schemes, inclusion criteria and end-points investigated, so that clear evidences are far from being depicted. In fact, attempts to synthetize clinical data into meta-analysis yielded to inconsistent results (Limketkai et al., 2020a; Iheozor-Ejiofor et al., 2020; Kaur et al., 2020). Nonetheless, utilization of *E. coli* Nissle 1917 for remission maintenance in UC patients and of VSL#3 probiotic mixture in pouchitis is indicated as possible options in international guidelines (Harbord et al., 2017; Su et al., 2020), suggesting that well designed clinical trials would ideally expand utilization of more probiotic species in specific IBD setting and indications. Indeed, a very recent study brilliantly highlighted that the variable results of probiotics in human studies may be related to two conceptual shortcomings: first, the fact that most studies rely on fecal, rather than mucosal, probiotic concentration as a marker of colonization, and second, the lack of appropriate investigation of the subjects' microbiota before probiotic administration, since different composition has been found to be related to a "permissive" or "resistant" phenotype to exogenous bacteria administration (Zmora et al., 2018).

Vitamin D/VDR and Immune System Regulation in IBD

Vitamin D is a fat soluble secosteroid hormone that can be assumed in the diet in two forms: vitamin D2 (ergocalciferol), present in mushrooms and vegetables, and vitamin D3 (colecalciferol), in fish and meet. The alimentary source is substantially scarce, and vitamin D3 is endogenously synthetized in the skin for the transformation by the UV light of the cholesterol precursor 7-dehydrocholesterol in pre-vitamin D3 and then in vitamin D3. In the blood stream, vitamin D3 and D2 are converted by a double hydroxylation process in the liver, by the enzyme 25-hydroxylase (CYP2R1) in 25 hydroxyvitamin D (25(OH)D), and in the kidney, by the enzyme 1-α-hydroxylase (CYP27B1), into its active form, 1,25-dihydroxyvitamin D (1,25(OH)2D or calcitriol). VDR is a single aminoacidic chain polypeptide of the nuclear receptors superfamily, and it is widely and differently expressed in many tissues, including intestinal mucosa and immune cells (Pike et al., 2017). The binding of 1,25(OH)2D to VDR in the cytoplasm of the cell, with the heterodimerization with the retinoid X receptor (RXR), determines the translocation of the complex to the nucleus and the binding to vitamin D response elements (VDREs), with stimulation and/or suppression of gene transcription (Pagnini et al., 2021). The biologic action of vitamin D/VDR signalling, initially characterized in the bone metabolism, is pleiotropic, and the correct functioning of this pathways has a paramount role for homeostasis maintenance at several levels. Multiple molecular effects may have a positive role in preventing and ameliorating chronic intestinal inflammation in IBD patients, and in particular the enforcement of intestinal barrier, the immunomodulation, and the microbiota modulation (Kellermann et al., 2020). In fact, experimental data indicate that vitamin D/VDR signalling stimulates functionality of tight junction proteins. VDR knockout and vitamin D-deficient mice showed epithelial barrier impairment with hyperfunction of claudin-2, and increased susceptibility to invasive bacteria colonization and colitis (Assa et al., 2015; Zhang et al., 2019). Vitamin D supplementation showed beneficial in Dextran sulphate sodium (DSS) model of colitis, by preserving the expression of E-cadherin, claudin, and zonula occludens in Caco-2 cells (Zhao et al., 2012). At intestinal mucosal level, vitamin D/VDR interaction display immunoregulatory effect, with a global stimulation of innate defence and regulation of pro-inflammatory mediators of the acquired compartment of immunity (Kellermann et al., 2020). In fact, vitamin D induces a TLR2/1-dependent activation of cAMP and beta-defensin 2 expression in monocytes and macrophages, with an increased anti-microbial function, and a vitamin D deficient diet or a lack of VDR can determine impaired anti-bacterial activities of epithelial cells and increased inflammation (Liu et al., 2009; Wang et al., 2010). Experimental data demonstrate that vitamin D stimulates autophagy, that is an essential innate immune physiological mechanism by which potentially harmful antigens are cleared at the mucosal compartment, thus preventing gut inflammation and dysbiosis (Wang et al., 2010). Considering adaptive immunity, many data indicate that vitamin D inhibits Th1, Th17 cells, and DCs differentiation and promoting Treg cells, with a reduced production of pro-inflammatory cytokines [i.e., IL-17A, TNF-alpha, IL-6, and interferon-gamma (IFN-γ)] (Kamen and Tangpricha, 2010; Wang et al., 2010). The effect on innate immunity is probably the key for the modulation of intestinal microbiota by vitamin D. In a recent study in a mouse model with a lack of

expression of VDR in Paneth cells, Lu et al. elegantly demonstrated that VDR signalling is essential for bacteria recognition, pathogens clearance and dysbiosis prevention (Lu et al., 2021). In fact, vitamin D administration has demonstrate to influence microbiota composition in mice models and human studies, potentially preventing or correcting dysbiosis (Shang and Sun, 2017). In particular, a recent meta-analysis of fourteen studies confirmed a regulatory effect of vitamin D administration on intestinal microbiota composition, even though with mixed results (Waterhouse et al., 2019), and even studies investigating microbiota modification due to vitamin D in IBD patients showed conflicting results (Garg et al., 2018; Schaffler et al., 2018; Soltys et al., 2020). Considering clinical data, low vitamin D status has been found to be associated with a higher IBD risk and a recent meta-analysis, including nearly 1,900 subjects, showed that IBD patients had a 64% increased risk of vitamin D deficiency comparing with controls (Del Pinto et al., 2015). Even more recently, a meta-analysis including a total of 8,316 IBD patients (3115 UC, 5201 CD), showed that low 25(OH)D level was linked to higher risk of disease activity, mucosal inflammation, low quality of life (QOL) scores, and clinical relapse (Gubatan et al., 2019). Considering the low vitamin D levels and IBD occurrence/severity, the crucial question of whether it represents a cause or an effect remains still unsolved, even though recent observational studies examining vitamin D levels prior to the diagnosis of IBD seem to support the latter hypothesis (Opstelten et al., 2018; Limketkai et al., 2020b). Interventional studies investigating the effect of vitamin D supplementation in IBD patients are still preliminary and no clear evidence exists, but a recent meta-analysis of 18 studies, with a total of 908 IBD patients, indicated that vitamin D supplement significantly improved the 25(OH)D blood levels and, in seven trials, determined a consistent relapse rate reduction comparing with untreated patients (Li et al., 2018). Indeed, for the established role of vitamin D for the bone health and the high incidence of deficiency in IBD patients, periodic check and correction of insufficient levels is advisable in such patients, even though the administration for immunomodulatory purposes remains, at present, only a fascinating suggestion (Myint et al., 2020). Moreover, since the correction in deficient IBD patients appears rational and indicated, the beneficial effect of vitamin D supplement in patients with normal serum level is not straightforward and probably needs further investigation.

PROBIOTICS PLUS VITAMIN D: EVIDENCE FOR A SYNERGIC EFFECT

Molecular Interaction

Besides the aforementioned beneficial effect that probiotics and vitamin D may singularly exert in IBD patients, early experimental data are suggesting a possible direct interaction between those two nutraceuticals, that may confer increased anti-inflammatory effect in the intestinal mucosa. In fact, studies in VDR knock-out (KO) mice have shown a defective autophagy and presence of dysbiosis, with reduction of Lactobacilli and Bacteroidetes species, comparing with wild-type mice (Ooi et al., 2013). In experimental model of colitis, supplementation of butyrate stimulate VDR genetic expression and protein production, with amelioration of the colonic inflammation (Wu et al., 2015b), even though the exact contribution of the VDR pathway for the anti-inflammatory effect of butyrate is not completely elucidated, considering the concomitant activation of the cell surface G-protein coupled receptors (GPCRs) such as GPR41, GPR43, and GPR109A, potentially involved for the immunomodulatory effect of butyrate in intestinal mucosa (Parada Venegas et al., 2019). Moreover, recent studies demonstrated that VDR functioning pathway is necessary for probiotics protection against colitis. In an elegant study, Wu et al. demonstrated that *Lactobacillus rhamnosus* GG and *Lactobacillus plantarum* stimulated VDR expression and activity in different cell lines, and that the administration of the two probiotic bacteria had a protective effect against Salmonella-induced colitis only in wild-type mice with intact functioning of the VDR pathway, while that protective effect was completely abrogated in VDR knock-out (KO) mice (Wu et al., 2015a). In addition, further experimental data demonstrated that probiotics stimulate VDR expression and activity. In the trinitrobenzene sulfonic acid (TNBS) inflammation-cancerogenesis model, the administration of the multiple probiotic compound VSL#3 stimulated VDR expression (together with angiostatin and alkaline sphingomyelinase), thus delaying the inflammatory mediated transition to dysplasia and cancer (Appleyard et al., 2011). The same multi-species probiotic product has shown to induce expression and modulate activity of VDR and other nuclear receptors, in an animal model of genetic dyslipidemia, with a reduction of insulin resistance in liver and adipose tissues and protection against development of steatohepatitis and atherosclerosis (Mencarelli et al., 2012). Early administration of *Lactobacillus casei* BL23 in larval zebrafish positively influenced growth, immune system development and survival, by means of induction of genes with different involvement in homeostasis, among which VDR-α (Qin et al., 2018). As a further confirmation of the strain-specificity properties of probiotic bacteria, among six Lactobacillus strains tested, only *L. plantarum* significantly induced VDR expression in HT-29 MTX cells (Raveschot et al., 2020), even though the association between increased expression of VDR and its activity is still not fully demonstrated. Besides the effect on VDR, some clinical and experimental data indicate that probiotic bacteria may increase vitamin D levels. In a post-hoc analysis of a randomized controlled trial investigating the cholesterol-lowering efficacy of the bile salt hydrolase active *Lactobacillus reuteri* NCIMB 30242, surprisingly, the probiotic bacteria did not impaire the absorption of fat-soluble vitamins, and yet increased the mean circulating level of 25-Hydroxyvitamin D, after 9 weeks of administration (Jones et al., 2013). In clinical studies including patients after bariatric surgery, administration of a multiple probiotic compound, from 4 weeks prior to 12 weeks after surgery, increased 25-OH Vitamin D serum level in patients undergoing One Anastomosis Gastric Bypass- Mini Gastric Bypass (Karbaschian et al., 2018), and the same effect was observed for an association of *Lactobacillus acidophilus* NCFM and Bifidobacterium lactis Bi-07, administered for

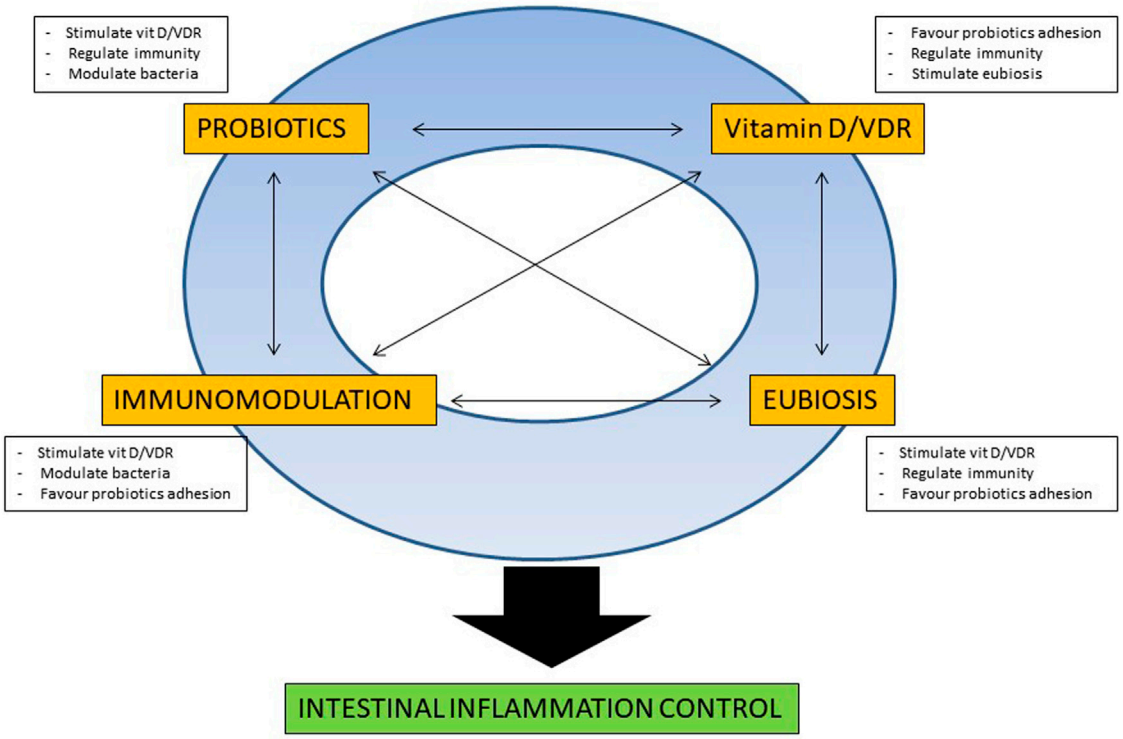

FIGURE 1 | Schematic representation of the possible reciprocal molecular interactions between probiotics and vitamin D for intestinal mucosal homeostasis. For a detailed explanation refer to the text.

3 months after Roux-en-Y Gastric Bypass (Ramos et al., 2021). In a computational modeling framework analysis, prebiotic stimulates pro-vitamin D3 by means of an increased production of lactate by stimulated Lactobacilli (Gokhale and Bhaduri, 2019). Although the exact molecular mechanism for the increased vitamin D by probiotics remains to be elucidated, possible factors are the increased absorption at intestinal level, mediated by increased ion concentration and lower pH, the increased substrate concentration, given by the lactate produced by the probiotic bacteria, and the activity stimulation of key enzymes of the vitamin D pathway, such as hepatic 25-hydroxylase or hepatic 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (Hollander et al., 1978; Yavuz et al., 2009). Therefore, considering experimental data, a hypothetical model for probiotic/vitamin D interaction for their beneficial effect in IBD patients could be drawn, as represented in **Figure 1**. In a virtual circle with multiple reciprocal interactions, specific probiotic bacteria may increase circulating vitamin D levels and stimulate the mucosal expression and activity of VDR, that in turn may exert immunomodulation of the mucosal immunity, with an enforcement of innate and anti-bacterial defences and a reduction of Th1 polarized cytokines, with a global anti-inflammatory mucosal effect. The stimulation of the innate response contributes to positively regulate the intestinal microbiota and to resolve or prevent dysbiosis, further favouring temporary colonization of administered probiotic bacteria and the stimulation of proliferation of butyrate-producing bacteria, with a consequent activation of vitamin/VDR pathway in a looping manner.

Clinical Data

Despite mounting data on potential biological interaction between vitamin D and probiotics, clinical data are still at the beginning. To date, ten randomized clinical trials (RCTs) (Savino et al., 2015; Tazzyman et al., 2015; Miraglia Del Giudice et al., 2016; Jafarnejad et al., 2017; Raygan et al., 2018; Ghaderi et al., 2019; Jamilian et al., 2019; Ostadmohammadi et al., 2019; Hajipoor et al., 2021; Morvaridzadeh et al., 2021), investigating the application of co-administration of vitamin D and probiotics, has been published (**Table 1**), seven of which have been included in a recent systematic review (Abboud et al., 2020). No clinical trial investigated so far the simultaneous application of probiotics and vitamin D in IBD patients. Waiting for clinical data, utilization of those nutraceuticals appears rational and may already be proposed as a supportive treatment in induction and maintenance of remission, as an ancillary therapy to the evidence-based treatments currently approved and available. In fact, considering the safety profile and the rational for their utilization, they may contribute to increase treatment efficacy and improve the management of IBD patients. Encouraging clinical data comes from different settings, and nearly all the published studies demonstrated a beneficial effect of probiotics and vitamin D co-administration. Nonetheless, results need to be taken with great caution, and clinical data in this field have to be considered preliminary. In fact, a consistent dishomogeneity exists in published studies, since trial designs, therapeutic schemes, probiotic species, probiotics/vitamin D doses,

TABLE 1 | Randomized clinical trials (RCTs) investigating the effect of co-administration of probiotics and vitamin D in different clinical conditions; no trial, at present, evaluated the effect of probiotic plus vitamin D in IBD patients.

Study (first author, year)	Disease	N	Vitamin D dose	Probiotic species	Comparator	Outcome
Ghaderi (2019)	Schizofrenia	60	50,000 IU/2 weeks	*L. acidophilus*, *B. bifidum*, *L. reuteri*, *L. fermentum*	Placebo	Beneficial
Jafarnejad (2017)	Osteopenia	50	200 IU/day	*L. casei*, *B. longum*, *L. acidophilus*, *L. rhamnosus*, *L. bulgaricus*, *B. breve*, *S. thermophilus*	Vitamin D alone	Some molecular difference but no effect on BMD
Jamilian (2019)	Gestational diabetes	87	50,000 IU/2 weeks	*L. acidophilus*, *B. bifidum*, *L. reuteri*, *L. fermentum*	Probiotic alone; placebo	Beneficial
Ostadmohammadi (2019)	Polycystic ovary syndrome	60	50,000 IU/2 weeks	*L. acidophilus*, *B. bifidum*, *L. reuteri*, *L. fermentum*	Placebo	Beneficial on mental health but no effect on other parameters
Raygan (2018)	Type 2 diabetes	60	50,000 IU/2 weeks	*L. acidophilus*, *B. bifidum*, *L. reuteri*, *L. fermentum*	Placebo	Beneficial on mental health, glycemic level, HDL, CRP but no effect on other metabolic profiles and hypertension
Savino (2015)	Infantile colic in newborns	105	400 IU/day	*L. reuteri* DSM 17938	Vitamin D alone	Beneficial
Tazzyman (2015)	IBS	51	3,000 IU/day	*L. acidophilus*, CUL 60, CUL 21, *B. bifidum* CUL 20, *B. animalis* sub. Lactis CUL 34	Vitamin D alone+placebo; placebo+placebo	No effect
Miraglia Del Giudice M (2016)	Asmatic allergic children	32	400 IU/day	*L. reuteri* DSM 17938	Placebo	Beneficial
Hajipoor S (2021)	Obese	140	1,000 IU/day	*L. acidophilus* La-B5, *B. lactis* Bb-12	1)Plain yogurt, 2) yogurt+probiotics alone, 3) yogurt+vitamin D alone	No difference in lipid profile, anthropometric indices
Morvaridzadeh M (2021)	NAFLD	104	1,000 IU/day	*S. thermophilus*, *L. bulgaricus*, *L. acidophilus* La-5, *B. lactis* Bb-12	Plain yogurt	Beneficial on 25(OH)D3 level, no effect on blood sugars and antropometric parameters

L. , – Lactobacillus, B. – Bifidobacterium, IBS – irritable bowel syndrome, NAFLD – non-alcoholic fatty liver disease.

duration of treatments, clinical settings, and sample sizes profoundly differ. Among published trials, only one investigated a gastroenterological condition, namely irritable bowel syndrome (IBS) (Tazzyman et al., 2015). No significant difference in symptoms was observed between patients who had co-supplementation with probiotics and vitamin D, compared with those who had vitamin D alone, or placebo. However, this study had a limited sample size and a limited duration of follow-up, and presented a consistent placebo effect, which may be due to different sun exposure between the investigated groups. Among the tested pathologic conditions, particularly positive results of vitamin D/probiotics administration has been observed in metabolic disorders, potentially representing a promising path for future research.

FUTURE PERSPECTIVES AND CONCLUSION

Despite pre-clinical data for a possible interaction of vitamin D/VDR pathway and probiotic administration in ameliorating intestinal inflammation, clinical studies are still to come. Considering the encouraging clinical data from other clinical settings, this therapeutic option appears intriguing and promising and deserve future investigation. In order to design reliable trials, flaws emerged from pre-clinical and clinical studies in probiotics and vitamin D application in IBD needs to be taken into account and carefully addressed. First, the choice of the probiotic bacteria appears to be relevant, considering that beneficial properties may differ even at strain level. A well studied bacterial species, with solid safety data and documented anti-inflammatory effect in the intestinal mucosa, could most probably lead to better results. Moreover, pre- and post-interventional assessment of microbiota quali-quantitative composition, together with the verification of temporary mucosal colonization of the supplemented probiotic, by means of genomic-based techniques, may provide further insights into potential mechanism of action of nutraceuticals, pre-selection of patients, and identification of potential markers for efficacy evaluation. Considering the high rate of vitamin D deficiency, and the lack of specific target levels for IBD patients, assessment of pre- and post-interventional blood levels, and evaluation of VDR mucosal expression, could help in identifying surrogate markers to pre-stratify patients and to monitor and guide nutraceutical supplementation modalities. In this regard, the possible presence of polymorphism of VDR genes (namely, TaqI and FokI), described in up to 20% of IBD patients, that may influence VDR functionality and therefore potentially reduce the response to vitamin D administration (Xue et al., 2013), need to be probably assessed. Finally, considering the variability

of the clinical pictures that fall under the term of "IBD," it is necessary to design interventional studies in specific restricted homogeneous clinical condition, as for example UC patients with proctosigmoiditis or CD patients with inflammatory phenotype and exclusive ileal localization. Moreover, possible confounding factors, such as diet, sun exposure, metabolic status, co-morbidities, and drug utilization, should be carefully assessed and standardized. In conclusion, vitamin D/probiotics co-administration appears a rational and attracting therapeutic option in IBD patients, but clinical data do not exist yet. The appropriate design of reliable trials will help to evaluate the potential efficacy, identify specific conditions and administration modalities, that would support and propose the contemporary supplement of vitamin D and probiotic in clinical practice for IBD patients.

AUTHOR CONTRIBUTIONS

CP and GD conceived the paper, CP wrote the paper, MD and MG revised the paper.

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REVIEW
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Possible Benefits of *Faecalibacterium prausnitzii* for Obesity-Associated Gut Disorders

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Metabolic disorders are an increasing concern in the industrialized world. Current research has shown a direct link between the composition of the gut microbiota and the pathogenesis of obesity and diabetes. In only a few weeks, an obesity-inducing diet can lead to increased gut permeability and microbial dysbiosis, which contributes to chronic inflammation in the gut and adipose tissues, and to the development of insulin resistance. In this review, we examine the interplay between gut inflammation, insulin resistance, and the gut microbiota, and discuss how some probiotic species can be used to modulate gut homeostasis. We focus primarily on *Faecalibacterium prausnitzii*, a highly abundant butyrate-producing bacterium that has been proposed both as a biomarker for the development of different gut pathologies and as a potential treatment due to its production of anti-inflammatory metabolites.

Keywords: diabetes, gut permeability, obesity, probiotics, *Faecalibacterium prausnitzii*

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INTRODUCTION

Obesity has increased worldwide (Di Cesare et al., 2016) and is a public health concern across the globe. This disease is associated health outcomes such as diabetes, hypertension, and cancer (Arroyo-Johnson and Mincey, 2016). Currently, it is estimated that 30% of the global population is overweight, and this number continues to increase (Talukdar et al., 2020). The most prevalent consequence of obesity is insulin resistance and the development of type 2 diabetes (T2D). This condition is driven by inflammation that begins in adipose tissue. The hyperglycemia associated with obesity is linked with gut inflammation, increased permeability to bacterial products, and changes in microbiota composition that promote the cycle of inflammation associated with the obese state (Fallucca et al., 2014). Such pathogenic modifications of the community structure of gut microbiota are referred to as dysbiosis; one of the most well-known examples in the context of obesity is an increase in the ratio of Firmicutes to Bacteriodetes in both mice and humans, which produces a pro-inflammatory profile characteristic of the obese microbiota (Kim et al., 2012; Verdam et al., 2013).

Diet has a direct influence on intestinal inflammation, which is related to weight gain and microbiota changes. A few weeks of a high-fat, high-sugar diet is enough to induce certain gut disorders, including increased gut permeability and dysbiosis (Cani et al., 2001b). After this it is possible to detect free lipopolysaccharides (LPS) in the blood, which triggers the activation of the innate immune system and inflammatory conditions (Guerville et al., 2017).

The development of hyperglycemia has been linked with a variety of alterations in the gut mucosa in addition to dysbiosis. These include increased gut permeability, altered expression of tight junction molecules, activation of innate immune molecules, and an increase in activated adaptative immune cells. All those changes characterize a "leaky gut," the terminology used to collectively describe those gut alterations (De Kort et al., 2011). Together, these conditions can perturb gut homeostasis and increase the probability of developing an inflammatory disease such as Crohn's disease.

Currently, the best diet-based strategy for treating these conditions is a shift from a Western-style diet to a balanced, plant-based diet. However, the implementation of such changes takes time and requires a very high level of nutritional education. Because of this, the success rates of weight loss maintenance due to lifestyle changes are typically low (Lewis and Abreu, 2017). In response, alternative treatments are being developed that aim to ameliorate the negative gut effects. One group of treatments that has demonstrated promise are probiotics, which in certain cases have been shown to decrease both gut permeability and glycemia (Barengolts, 2016; Xu et al., 2019).

Faecalibacterium prausnitzii (F. prau) is a probiotic isolated from the human microbiota, where it is a dominant species in healthy adults. Its decline is associated with the development of chronic inflammation, as observed in cases of obesity. Multiple studies have demonstrated the anti-inflammatory properties of this bacterium (Feng et al., 2014; Andoh et al., 2016; Ganesan et al., 2018; Wrzosek et al., 2018), which are thought to be associated with its ability to produce butyrate. Butyrate activates the G-protein receptor (GPR) and thus facilitates downstream control of gut alterations during obesity and diabetes (Brown et al., 2003). Therefore, the goal of this review is to discuss the current state of knowledge regarding *F. prau*, particularly with respect to its potential as probiotic derived from human gut for use in alleviating the gut inflammation developed during obesity and hyperglycemia.

OBESITY AND HYPERGLYCEMIA-ASSOCIATED GUT ALTERATIONS

Obesity is a highly complex, multifaceted disease associated with numerous metabolic dysfunctions. These include type 2 diabetes mellitus (T2DM), dyslipidemia, cardiovascular dysfunction, and chronic inflammatory diseases (Winer et al., 2016). These conditions are linked with changes in adipose tissue (AT), a complex endocrine organ that plays an important role in energy homeostasis due to its rapid and dynamic responses to changes in nutrient availability (Sun et al., 2011). Adipose tissue is composed of adipocytes, macrophages, lymphocytes, fibroblasts, cell progenitors, and endothelial cells, and is responsible for the secretion of molecules such as leptin, adiponectin, cytokines, and the vascular regulators angiotensin II and plasminogen activator inhibitor (PAI-1) (Andersen et al., 2016).

In conditions of obesity, AT can become severely dysfunctional, with changes ranging from an increase in size to impaired function and atypical distribution in the body. This results in a suite of physiological alterations, including modifications to the extracellular matrix, vascularization, levels of oxidative stress, the profile of secreted adipokines, and the inflammatory state of infiltrated immune cells (Jo et al., 2009). Due to the increase in free fatty acids (FFA), signaling pathways such as IKKβ and NF-κB are activated, along with those linked with Toll-like receptors (TLRs) (Crawford et al., 2009; Baker et al., 2011), which influence the inflammatory state. Obese patients are typically characterized by an increase in LPS that is accompanied by higher levels of TLR4 and CD14 expression, which all contribute to the proliferation of pro-inflammatory mechanisms (Winer et al., 2016).

The activation of TLRs is important in obesity, especially TLR4 (Winer et al., 2016), as these signaling pathways regulate the phosphorylation of proteins and lead to an increase in the production of molecules such as TNF-α, IL-6, leptin, resistin, and chemokines like type 2 CC chemokine receptor (CCR2), which is related to monocyte migration. TNF-α promotes the activation of the NF-kB pathway, stimulation of the cell death signaling pathway, inhibition of the expression of the glucose transporter GLUT-4, and an increase in FFA levels and consequent reduction in insulin sensitivity (Gomez-Hernandez et al., 2016).

Compared to their lean counterparts, mice that are fed a high-fat diet present a higher number of $TCD4^+$ and $TCD8^+$ cells and higher levels of IFN-γ and TNF-α, mainly in adipose tissue (Lumeng et al., 2007). The infiltration of $TCD8^+$ cells in adipose tissue is followed by the accumulation of $CX3CR1^{int}$ macrophages, which migrate towards AT in response to greater amounts of FFAs, glucose, and apoptosis, thus increasing inflammation (Rocha et al., 2014). Adipose tissue macrophages (ATMs) infiltrate adipose tissue in a CCR2-dependent manner and inhibit the insulin signal in insulin-sensitive tissues, including liver, adipose tissue, and muscle (Hotamisligil, 2017; Kawano et al., 2016). Taken together, these conditions contribute to the production of pro-inflammatory cytokines and the release of monocyte 1 and 3 chemotactic protein (MCP-1 and MCP-3), which creates a cycle of continuous cell recruitment and constant inflammation in AT (Nishimura et al., 2009).

The inflammatory state in visceral adipose tissue, along with an excess of metabolites in the circulation, is a major driver of obesity-related insulin resistance (IR). IR is characterized by impaired phosphorylation of the insulin receptor in cells that depend on insulin. This results in increased serine phosphorylation of insulin receptor substrate 1 and 2 (IRS-1 and IRS-2) and activation of the SOCS (suppressor of cytokine signaling) protein, which reduces the insulin receptor's ability to transmit signals downstream in the insulin pathway and to capture glucose in cells (Biddinger and Kahn, 2006).

When the full extent of metabolic dysfunction is considered, the physiological impact of obesity is wide-ranging, with numerous effects on the architecture and functionality of primary and secondary immune system organs, including bone marrow, thymus, and lymph nodes (Yang et al., 2009). The accumulation of lipids reduces hematopoiesis in bone marrow and thymopoiesis in the thymus, which is added to a restricted diversity of T cell receptor repertoires in the thymus

(Yang et al., 2009). In the peripheral immune response, there is a reduction in the migration of antigen-presenting cells to peripheral lymph nodes and a consequent reduction in the differentiation of naïve T cells into effective cells. These systemic effects can also translate into disturbances to the homeostasis of gut mucosa, which together with the mucosal-associated lymphoid tissue harbor the majority of immune cells found in the body.

The intestinal mucosa is the largest surface of the human body that is in contact with the external environment (Rezende and Weiner, 2017). The intestinal immune system is thus faced with an immense challenge: tolerating the vast amount of antigens that originate from the diet and the commensal microbiota while, at the same time, protecting against intestinal pathogens and toxins (Faria et al., 2017). Alterations in the intestinal barrier and dysbiosis in the gut of obese individuals may not only favor the development of diseases, but may also compromise immune tolerance to dietary antigens and the microbiota (Spiekermann and Walker, 2001; Faria and Weiner, 2005).

In the literature, there is an abundance of evidence on the many ways obesity and insulin resistance can affect immune responses and gut physiology. Obesity enhances jejunal inflammation and increases the density of macrophage populations, CD3+ T cells, intraepithelial lymphocytes and mature dendritic cells. It also increases the expression of pro-inflammatory cytokines (IFNγ, IL1β, TNFα), chemokines, and co-stimulatory factors in cells from the lamina propria and epithelial compartment. The increase in T-cell populations in the intestinal mucosa of obese subjects has also been associated with an impaired insulin response compared with lean subjects (Monteiro-Sepulveda et al., 2015). In humans with obesity and insulin resistance, there is a stronger inflammatory profile in the duodenum compared to non-obese patients, with more inflammatory cytokines and M1 macrophages (Ho-Plagaro et al., 2019). In addition, obesity and excess weight have been associated with increased gut permeability—demonstrated by increased serum concentrations of zonulin—along with microbiota modifications (Mörkl et al., 2018). Increased gut permeability was also reported in individuals with high levels of fasting glucose, as well as higher levels of pro-oxidative markers in the blood compared with healthier subjects (Carnevale et al., 2017). Furthermore, intestinal permeability was found to be sharply increased by a high-fat diet (HFD), probably mediated by a reduction in the expression of ZO-1 and occludin, which then favors the translocation of LPS through the intestinal wall (Cani et al., 2008b).

Hyperglycemia associated with HFD increases levels of IFN-γ– and IL-17–producing inflammatory cells in the intestine and intestinal permeability, and decreases the abundance of regulatory cells (Luck et al., 2015). Even in the absence of excess weight, hyperglycemia has been associated with increased permeability and alterations in the mucosal immune system. For example, in *db/db* mice with controlled food intake or in mice treated with streptozotocin, high levels of blood glucose were associated with high intestinal permeability and increased expression of PRP (pathogen recognition patterns) in lymphoid organs (Thaiss et al., 2018). In other words, hyperglycemia that is secondary to obesity, IR, or even T1D can further compromise intestinal health. Alterations in glucose metabolism can also decrease oral tolerance of antigens from the diet (Miranda et al., 2019) and increase the severity of food allergies (data not published) in mice.

One of the main consequences of the development of inflammatory alterations and the breakdown of gut epithelial integrity is the leakage of bacterial products, including endotoxins such as LPS, across the intestinal epithelium. This then results in dysfunction of immune organs, inadequate distribution of leukocyte populations, and changes in lymphocyte activity, which can all affect the immune response against pathogens, regulation of the immune response to dietary antigens, and the intestinal microbiota (**Figure 1**).

CHANGES IN GUT MICROBIOTA RELATED TO OBESITY AND HYPERGLYCEMIA

Composition of the microbiota is modulated by the availability of dietary nutrients that provide a wide variety of essential metabolites for the maintenance of intestinal architecture and integrity, while simultaneously acting on the modulation of immunity. In healthy conditions, Bacteroidetes and Firmicutes are the most abundant phyla of the microbial gut community. Although the structure of these communities can vary, some general patterns have been noted. For example, studies have determined that some of the most abundant intestinal bacterial species in healthy individuals tend to include members of the *Dorea/Eubacterium/Ruminococcus* groups as well as Bifidobacteria, Proteobacteria, and streptococci/lactobacilli (Eckburg et al., 2005; Qin et al., 2010).

Diet is the key determinant of microbiota composition; it modulates the abundance of various species and, consequently, their individual or collective functions. In humanized gnotobiotic mice, a shift from a low-fat, plant polysaccharide–rich diet to a high-fat and high-sugar diet had detectable effects on microbial community structure and metabolic pathways after only a single day. More specifically, the increase in body fat percentage in mice fed a high-fat diet was positively associated with the abundance of species in the genera *Lactococcus* and *Allobaculum* but was negatively associated with *Akkermansia* (Kolodziejczyk et al., 2019).

The alterations in the microbiota linked with HFD-induced obesity also have effects on gut permeability to bacteria and bacterial products. The Burcelin group (2008, 2011) described that after 1 week of HFD consumption, changes could be seen in the intestinal mucosa and microbiota such as co-localization of bacteria with dendritic cells (DCs) both in the mucosa and in the mesenteric lymph nodes. After 4 weeks of HFD consumption, clear increases in intestinal permeability have been noted, with a concomitant decrease in zonulin expression in the intestine. These changes were also dependent on changes in the microbiota (Cani et al., 2008b; Amar et al., 2011). These alterations in the intestine permit increased bacterial translocation, which involves intestinal phagocytes and requires the recognition of pathogen-associated molecular

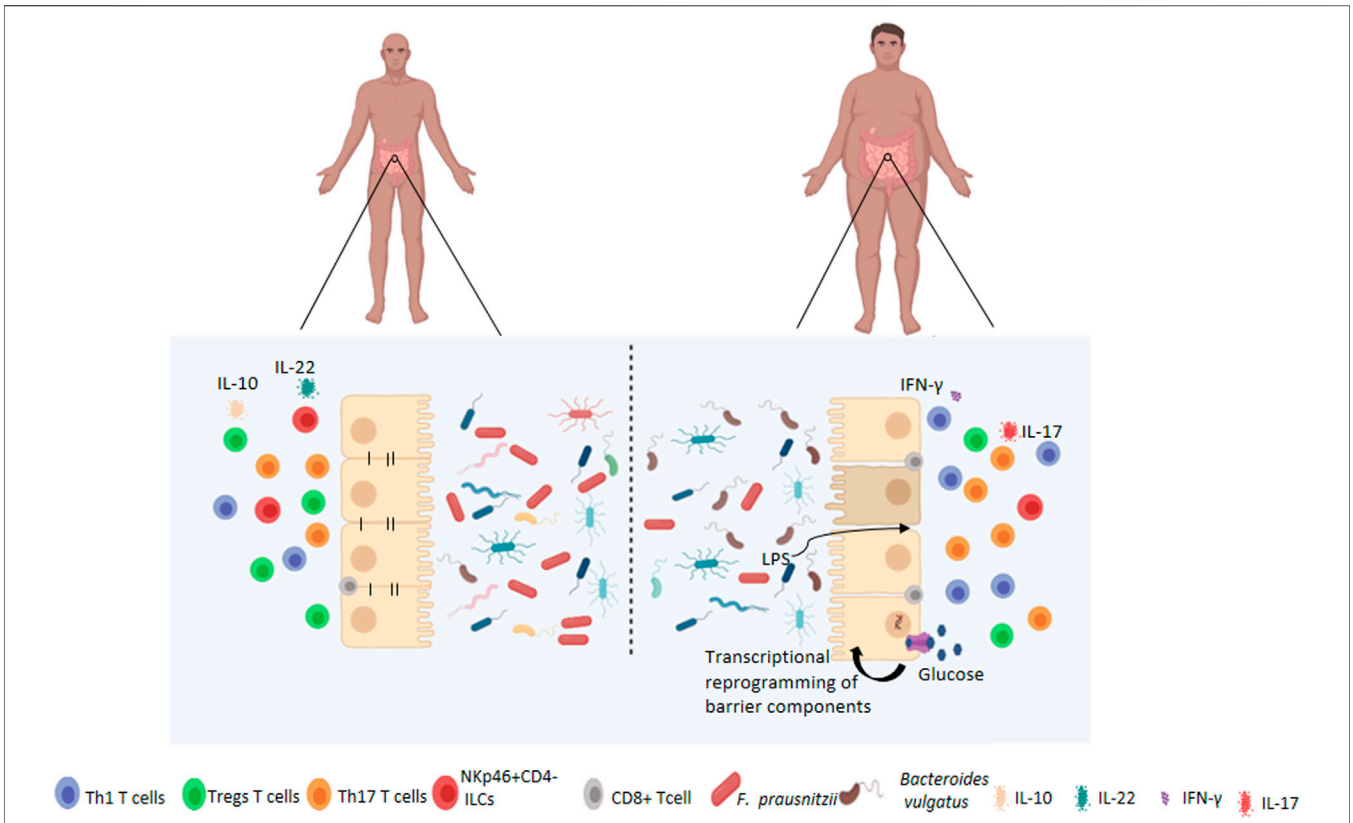

FIGURE 1 | Changes to the intestinal barrier functions associated with obesity. Under normal circumstances, the gut microbiota is highly diversified which contributes to the maintenance of intestinal permeability, tolerance to dietary antigens and immunoregulation. Obese patients have dysbiosis of the gut microbiota, characterised by decreased diversity, and altered barrier functions such as increased permeability and activation of pro-inflammatory pathways by immune cells at local and systemic levels. Obesity-associated hyperglycemia may also drive barrier permeability through transcriptional reprogramming resulting in decreased expression of adherens and tight junction proteins.

patterns (PAMPs) by standard recognition receptors (PRRs), such as TLRs and nod-like receptors, to activate an innate immune response (Kim et al., 2012; Luck et al., 2015). Under pathological conditions. However, this molecular signaling process can favor further infiltration and cell accumulation in adipose tissue, triggering local inflammation.

The relationship between dysbiosis and gut permeability also plays a role in the development of insulin resistance. Specifically, alterations in the abundance of intestinal bacteria caused by chronically elevated glucose levels and obesity are known to result in dysfunction in the intestinal barrier (Guerville et al., 2017). As a result, high concentrations of gram-negative bacterial cell-wall products, such as LPS, can cross the intestinal barrier to reach other organs and tissues, and induce chronic inflammation (Cani et al., 2009). This can initiate a series of inflammatory mechanisms, setting in motion one of the main processes that leads to insulin resistance and ultimately T2D (Cani et al., 2007; Ganesan et al., 2018). This can be further exacerbated by the fact that the production of pro-inflammatory cytokines interferes with insulin secretion and the expression of insulin mRNA in human beta islet cells. Changes in the composition of the microbiota can thus play a multi-faceted role in intestinal barrier dysfunction and, consequently, in metabolic disorders.

Certain groups of bacteria have been linked with various conditions in the gut. As mentioned earlier, an increased ratio of Firmicutes to Bacteroidetes in obese individuals has been related to inflammatory diseases (Turnbaugh et al., 2006), while conversely, the ratio of *Bacteroides* to *Prevotella* is lower in obese subjects than their lean counterparts. *F. prau* has been negatively associated with insulin resistance (Furet et al., 2010), while potential proinflammatory bacteria such as *Ruminococcus gnavus* or *Bacteroides* may dominate the microbiota of obese patients. In general, a reduction of butyrate-producing bacteria in obese subjects has been associated with an increase in mucus degradation (Cotillard et al., 2013). Obese women with high serum levels of zonulin, which is correlated with higher gut permeability, showed decreased abundance of Ruminococcaceae and *Faecalibacteri* both could weaken the gut barrier and lead to systemic inflammatory responses (Mörkl et al., 2018).

A study in mice reported that, after 1 week of HFD, and the subsequent early onset of diabetes, gram-negative bacteria start to adhere in the DCs of the gut mucosa; this increased bacterial

translocation and triggered inflammation (Amar et al., 2011). Interestingly, mice that are deficient in TLR5, which recognizes bacterial flagellin, develop obesity and features of metabolic syndrome even in the absence of a high-fat diet, solely as a function of microbiota changes (Vijay-Kumar et al., 2010). Finally, some bacterial species have been identified as particular promoters of insulin resistance, for example, *Prevotella copri* and *Bacteroides vulgatus*, which were noted to be the main species driving the association between the biosynthesis of branched-chain amino acids and insulin resistance in humans (Pedersen et al., 2016).

One important function of the microbiota is the metabolism of polysaccharides, through which these bacteria produce a wide variety of metabolites, such as short-chain fatty acids (SCFAs), that are essential for the microbial population and for the maintenance of intestinal homeostasis (Sun et al., 2017). The SCFAs acetate, butyrate, and propionate are the main products of fermentation in the intestine, and are of particular interest due to their ability to activate local G-protein-coupled receptors (GPCR) in epithelial cells, especially GPR41 and GPR43 (Miyamoto et al., 2016). This process has feedback effects on the physiology of the intestinal microbiome and mediates chronic inflammation, affecting both glucose homeostasis and insulin sensitivity (Ang and Ding, 2016; Lambertz et al., 2017). Butyrate-producing bacteria such as *F. prau* can reduce bacterial translocation and stimulate mucin secretion, which acts to maintain the integrity of the intestine (Ganesan et al., 2018).

Interactions between the intestinal microbiota and host cells require finely tuned control by the immune system, as specialized cells are needed to recognize bacterial fragments and induce inflammation when appropriate. A significant consequence of the microbial changes associated with obesity is activation of the innate immune system, which often results in chronic inflammation (Cani et al., 2007; Cani et al.,2008a). In animal models, increased levels of LPS in the bloodstream can directly damage pancreatic β cells and increase insulitis by triggering the innate immune response; this is a crucial factor in the pathogenesis of insulin resistance (Ganesan et al., 2018). Similarly, Amar et al. (2011) reported that excess LPS fragments in the blood of diabetic mice induced adipose tissue inflammation.

Interestingly, when HFD experiments are performed in animals lacking the microbial pattern recognition receptors Nod1 or CD14, diet no longer has strong effects on bacterial permeability and translocation (Amar et al., 2011). Likewise, TLR4-knockout mice have a completely different microbiota and display elevated blood LPS levels and diabetes development compared to WT controls. These alterations in gut morphology and microbiota composition are also correlated with lower levels of circulating SCFAs, which suggests an interaction between intestinal functions, microbiota composition, and the development of diabetes (Simon et al., 2020).

Taken together, the changes to the structure and function of the gut microbiota that occur in cases of obesity and hyperglycemia have the potential to severely exacerbate mucosal inflammation and gut permeability. For this reason, the use of bacterial probiotics—especially those known to produce high levels of SCFAs—could represent an interesting approach to treat the damage associated with intestinal metabolic syndrome.

PROBIOTICS AS AN ALTERNATIVE TREATMENT FOR OBESITY AND HYPERGLYCEMIA

Modulation of the microbiota has become a regular and effective approach in the treatment and prevention of mucositis, colorectal cancer, neurological diseases, and several other disorders (Sanders et al., 2019). There are several ways to modify the microbiota, including diet alteration; the administration of probiotics, prebiotics, and postbiotics; and fecal microbiota transplantation. The underlying goal of all of these methods is the same: to modify the bacterial composition in the gut in order to provide benefits to the host. As the links between obesity, hyperglycemia, and alterations in the microbiota have become clearer, studies have attempted to shed light on the exact mechanisms by which diet is able to affect the microbiome. It is possible to envision that microbiota modulation could serve as a non-invasive means of treating metabolic conditions, especially obesity, and thus represent an attractive alternative to common approaches such as bariatric surgery.

As mentioned above, obesity-related dysbiosis induces a series of events, mainly in the intestine, that result in chronic inflammation and disruptions to intestinal homeostasis. However, until the early 2000s, there was no discussion of the possibility that the microbiota itself could contribute to weight gain (Backhed et al., 2004). The Gordon group has conducted research on this topic, and their pioneering studies proposed an interesting hypothesis—that the microbiome from obese subjects has an increased capacity to harvest energy from the diet. First, it was reported that control mice colonized with an "obese" microbiota present higher body fat compared to mice colonized with "lean" microbiota (Turnbaugh et al., 2006). Further studies with gnotobiotic germ-free C57BL/6 mice revealed that a Western diet induced a drop in microbiota diversity, with an increase in abundance of a single class of Firmicutes. In addition, when the microbiota from conventionally raised obese mice were transplanted into wild-type mice, they showed a greater increase in body fat (Turnbaugh et al., 2008). Finally, a fascinating study with adult monozygotic and dizygotic twins showed that, although twins shared a core microbiota, obese siblings had less diversity in their microbial assemblages, lower proportions of Bacteroidetes, and a higher proportion of Actinobacteria compared to their leaner twins (Turnbaugh et al., 2009). These interesting findings have led to the generation of several hypotheses, with one of the most intriguing being that microbiota modulation by itself might be an effective treatment for obesity.

The relationship between gut microbiota and energy balance (and thus the development of larger adipose tissue) is complex because it involves many factors, such as diet, gender, and culture, among others. However, recent studies have pointed to the gut

microbiome as a key element in the regulation of food absorption; there is evidence that the gut microbiota can affect hormone secretion directly in the brain, in areas that are responsible for controlling appetite, fat storage, and energy expenditure (Cerdó et al., 2019). Although the science is far from settled on the topic, based on these initial results, some attempts have been made to treat obesity by modifying the host microbiota.

To date, probiotics and prebiotics have been used for the treatment of obesity in both experimental models and clinical trials. Probiotics are live microorganisms that, when ingested, can confer benefits on the host, while prebiotics are molecules or components (such as food fiber) that are capable of modifying the composition of the intestinal bacterial assemblage or stimulating the activity of one or a limited number of bacterial species in a positive way (Gibson et al., 2017). In most cases, one of the noted benefits of both prebiotics and probiotics is increased levels of SCFAs, which have regulatory effects and are important for intestinal integrity. Much research has focused on the positive effects of *Lactobacillus* and *Bifidobacterium* in obesity; both bacteria have been reported to contribute to weight loss and a reduction in insulin resistance. For instance, several studies involving different mouse or rat models of obesity have shown positive results from treatment with certain strains of *L. plantarum* (DSM 15313, Strain No. 14, and TL8), such as less weight gain, reduced adiposity, and higher insulin sensitivity (Axling et al., 2012; Ben Salah et al., 2013; Okubo et al., 2013). A recent publication reported that *L. plantarum* LMT1-48 significantly reduced weight in HFD-fed mice and its extract was capable of inhibiting the differentiation of adipocytes through downregulation of genes such as PPAR-γ (Choi et al., 2020). Notably, synergistic effects were observed from a probiotic combination of *L. plantarum* KY1032 and *L. curvatus* HY7601; HFD-fed mice treated with this mixture showed reduced body weight gain and fat accumulation, lower levels of insulin and cholesterol, and fewer biomarkers for inflammation (Park et al., 2013; Yoo et al., 2013). One exception was reported with HFD-fed mice treated with *L. plantarum* DSM 15313, which showed an increase in body weight despite lower levels of glucose in plasma (Andersson et al., 2010). Another study, with *L. plantarum* NCIMB8826, found no effect on body weight (Martinic et al., 2018). Overall, numerous strains—including *L. paracasei* CNCM I-4034, *L. casei* IMVB-7280, *L. paracasei* HII01, *L. casei* IBS041, *L. rhamnosus* CGMCC1.3, *L. rhamnosus* PB01 (DSM 14870), and *L. rhamnosus* LA68, among others—have demonstrated probiotic potential in studies of obesity, inducing positive effects such as reduced weight gain, less adiposity with less white adipose tissue, and reduced cholesterol levels, as reviewed by Ejathed et al. (Ejtahed et al., 2019).

Avolio and colleagues et al showed that HFD-fed hamsters treated with a probiotic mix of six species (*S. thermophilus, L. bulgaricus, L. lactis, L. plantarum, B. lactis*, and *L. reuteri*) presented a reduction in body weight and reduced levels of inflammatory factors in the blood compared to control HFD animals (Avolio et al., 2019). A study of leptin-deficient mice (ob/ob mice) showed that administration of the plant-derived lactic acid bacterium *Pediococcus pentosaceus* was sufficient to reduce adipocyte size and liver triglyceride content (Zhao et al., 2012). Similarly, HFD-fed mice treated with *L. plantarum* demonstrated reduced adipose tissue and triglyceride levels; the treatment also reduced the Firmicutes/Bacteroidetes ratio and improved the gut microbiota composition (Joung et al., 2021). Finally, although most effort to date has focused on bacteria, the most-studied yeast probiotic, *Saccharomyces boulardii,* has been reported to reduce hepatic steatosis and hepatic inflammation in db/db mice (Everard et al., 2014).

Positive results are also being reported from obese mice treated with various types of prebiotics. For example, the use of oligofructose as a prebiotic in HFD-fed obese mice resulted in higher numbers of intestinal *Bifidobacteria* and *Lactobacillus* in treated mice; this correlated with higher zonulin expression, leading to less intestinal permeability and less hepatic inflammation (Cani et al.,2008b). The addition of oligofructose in the diet was also found to promote satiety in HFD-fed mice, contributing to a reduction in both weight and adipose tissue deposits (Cani et al., 2005; Régnier et al., 2021). Another study showed that short-chain fructose-oligosaccharides induced a significant increase in the abundance of *Bifidobacteria* in obese mice, and these animals gained less weight than control HFD mice (Respondek et al., 2013). In general, the use of prebiotics as a treatment for obesity seems quite promising; this is especially true for approaches using food fiber, which seems to increase the production of satiety hormones, thus helping to control weight and increasing sensitivity to insulin (Parnell et al., 2012).

In humans, several clinical trials of probiotics and prebiotics as a treatment for obesity have reported positive results. For example, a randomized, double-blind study conducted by Osterberg and collaborators demonstrated that administration of a probiotic containing eight strains of bacteria (*Lactobacillus*, *Bifidobacterium*, and *Streptococcus*) attenuated increases in body mass index (BMI) and adipose tissue in subjects on a high-fat diet (Boutagy et al., 2015). Similarly, other studies of interventions with *Lactobacillus* spp. and *Bifidobacterium* spp. have reported positive results in obese and overweight subjects, such as less body weight and less fat storage (Minami et al., 2015; Madjd et al., 2017). Two recent systematic reviews concluded that some probiotic strains are effective in reducing BMI and hip circumference (Shirvani-Rad et al., 2021; Tomé-Castro et al., 2021).

Despite the promising results thus far, there is still much room for new approaches and improvements, especially those aimed at elucidating the mechanisms by which various probiotics and prebiotics induce reductions in weight and inflammation. In this context, the probiotic derived from human gut *F. prau* may prove particularly useful.

F. PRAU AS A NEW TREATMENT TO IMPROVE GUT HOMEOSTASIS DURING OBESITY AND IR

F. prau is a Gram-positive bacterium belonging to the Ruminococcaceae family, in *Clostridium* cluster IV. It is one of the most abundant species found in the gut, representing between 1 and 6% of the total fecal microbiota (Hold et al., 2003). Multiple

studies have described its anti-inflammatory properties and its role in tissue damage repair and protection against colitis(Sokol et al., 2008; Martín et al., 2014; Rossi et al., 2015), which appear to be related, at least in part, to its ability to produce butyrate(Zhou et al., 2018). *F. prau* is characterized by great intra-species diversity; indeed, it has been suggested that the genomic disparities are great enough to warrant separating the group into at least two different species: *F. prau* sensu stricto and *F. moorei* sp. nov (Fitzgerald et al., 2018).

Patterns of abundance of *F. prau* and its different phylogroups have been associated with various pathologies of the gut. For example, IBD patients were found to host reduced phylotype richness of *F. prau* compared to a healthy group. Specifically, total levels of *F. prau* phylogroup I were reduced, and this pattern could be used to accurately differentiate IBD and colorectal cancer patients from healthy subjects; instead, phylogroup II was specifically reduced in Crohn's disease (CD) patients. Interestingly, *F. prau* prevalence was found to be reduced locally in either the ileum, colon, or rectum depending on the form of the patient's CD (Lopez-Siles et al., 2016). Taken together, this demonstrates the potential applications of *F. prau* phylotypes as biomarkers for the diagnosis and prognosis of patients.

As discussed earlier, diversity of the microbiota is lower in obese patients, and *F. prau* has been implicated in these changes in community composition. For example, analysis of a Chinese cohort revealed a reduced abundance of *Bacteroides*, *Akkermansia* (another butyrate-producing bacterium), and *F. prau* in T2D patients. Evidence of this shift was even detectable in pre-diabetic obese patients, reflecting the link between glucose intolerance and microbiota composition (Zhang et al., 2013). These results—especially with respect to *F. prau*—were corroborated in a later Iranian study, which also reported a negative correlation between *F. prau* count and BMI (Navab-Moghadam et al., 2017). In another study, though, *F. prau* was found to be enriched in patients suffering from T2D after weight loss (Hippe et al., 2016). Thus, although the exact mechanisms have yet to be determined, the current state of research supports a link between BMI, blood glucose levels, and the abundance of *F. prau*.

Another interesting finding with respect to *F. prau* was the observation of differences in gut composition on the basis of gender, especially for this bacterium and streptococci. In a population of obese Chinese patients, a positive correlation was found between *F. prau* abundance and fasting glucose levels in men but not in women (Aguirre de Cárcer et al., 2011).

Rapid improvements (on the scale of a few days) have been observed in the community structure of gut microbiota as a result of nutritional interventions. Diets rich in non-digestible carbohydrates, such as inulin-type fructans, fructooligosaccharides, polydextrose, soluble corn fiber, and raffinose, have been observed to increase the abundance of *F. prau* (Verhoog et al., 2019). Supplements can also be effective; for example, kiwifruit-based supplementation was noted to increase *F. prau* abundance in the gut as well as stool frequency in humans (Rush et al., 2002; Blatchford et al., 2017).

Alternatively, supplementation with *F. prau* itself could have potential in the treatment of obesity and its associated disorders. HFD-fed mice that were treated twice a week with *F. prau* displayed decreased hepatic inflammation, with fewer lipids accumulated in the liver, as well as a reduction in cell infiltration of adipose tissue and adipocyte size compared to controls (Munukka et al., 2017).

Finally, many studies have examined the mechanisms by which *F. prau* exerts its beneficial impacts on the intestinal health of obese individuals (**Figure 2**). It appears that these effects may not only be a function of the abundance of this bacterium in the intestine, but also of the quantity and type of metabolites it produces, such as butyrate. Indeed, some of these metabolites have already demonstrated promise for the treatment of obesity and T2D (Ganesan et al., 2018; Verhoog et al., 2019).

F. PRAU ACTION THROUGH BUTYRATE PRODUCTION

F. prau is one of the most abundant butyrate-producing bacteria in human feces (Hold et al., 2003), and this SCFA is currently the subject of intense research focused on its positive health effects. For example, butyrate can prevent HFD-induced insulin insensitivity through epigenetic regulation that increases mitochondrial beta-oxidation, thus improving glucose sensitivity and adiposity (Fernandes et al., 2014).

The butyrate-containing supernatant of *F. prau* has been found to regulate Th17/Treg differentiation through inhibition of the IL-6 and STAT3/IL-17 proinflammatory pathway, specifically by targeting histone deacetylase 1 (HDAC1) (Rivière et al., 2016; Zhou et al., 2018). Although this mechanism was demonstrated in a colitis model, this proinflammatory pathway is also involved in obesity (Hippe et al., 2016). These results are consistent with the observation that HFD-mice treated with *F. prau* have improved hepatic health and reduced inflammation in adipose tissue (Munukka et al., 2017).

The anti-inflammatory properties of butyrate, and by extension *F. prau*, have long been known to be beneficial to IBD patients(JM et al., 1989; W et al., 1992; Scheppach, 1996). A recent clinical study found that administration of encapsulated sodium butyrate altered patients' gut microbiota by increasing the abundance of SCFA-producing bacteria in ulcerative colitis patients and butyrate-producing bacteria in Crohn's disease patients, with the former group reporting an increased quality of life (Facchin et al., 2020). Sodium butyrate supplementation also had positive effects on HFD-fed mice by altering the composition of the gut microbiota, lowering serum LPS concentration, and reducing HFD-induced inflammation (Zhou et al., 2017).

The A2-165 strain of *F. prau* has been found to induce a distinct cytokine response, with high IL-10 secretion compared to other *F. prau* strains tested (Rossi et al., 2016). This may be the result of higher butyrate production, which is known to induce IL-10 responses in Th1 cells (Sun et al., 2018). Indeed, a recent study demonstrated that this strain's anti-inflammatory

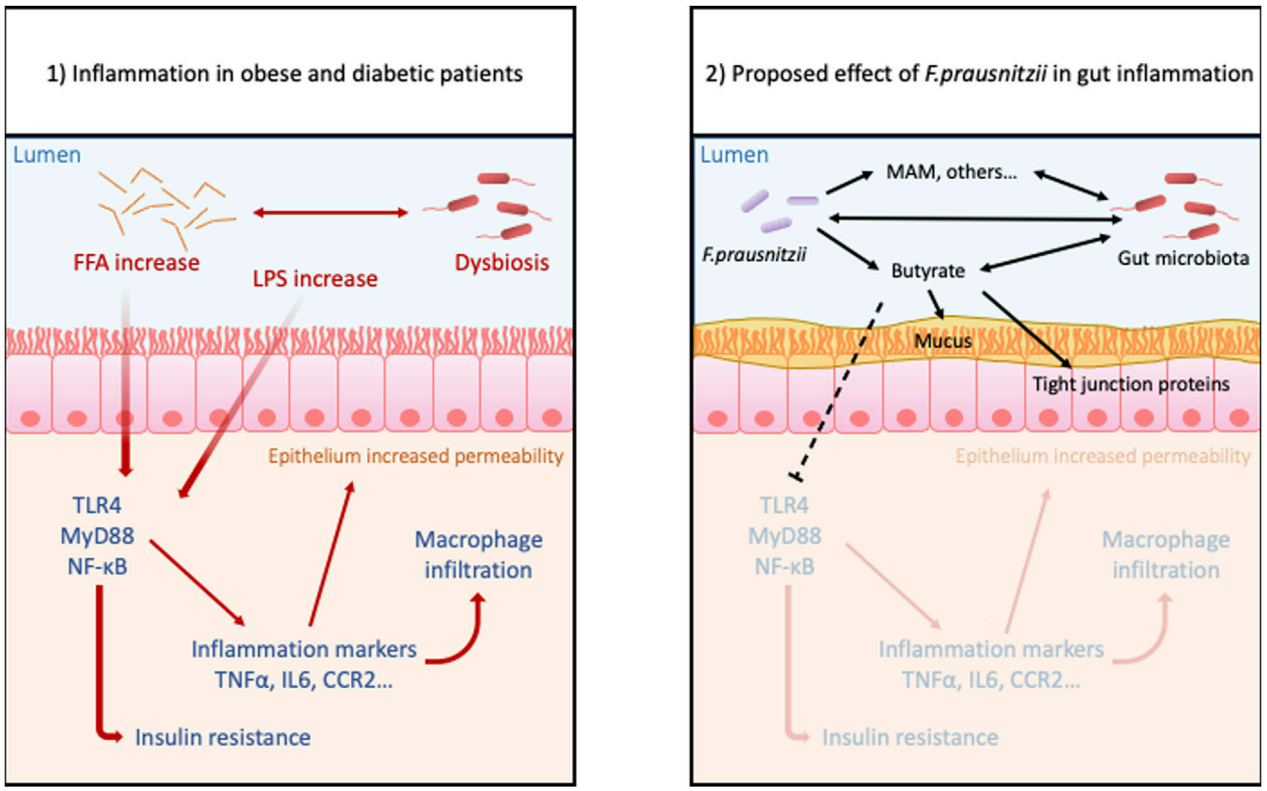

FIGURE 2 | Proposed benefits effects of *Faecalibacterium prausnitzii* in gut inflammation due to obesity: Obesity and hyperglycemia cause dysbiosis, increased level of FFA and LPS, and also disrupts intestinal permeability. These conditions trigger inflammatory pathways in the lamina propria allowing more permeability to antigens and bacteria. This leads to a leaky gut and susceptibility to inflammatory disorders. The treatment with probiotic F. prau and its products can increase the butyrate concentration, stabilizes the microbiota and mucous layer and decreases the activation of inflammatory pathway.

properties in primary human colonic mucosal barrier cells are primarily due to the downregulation of TLR3 and TLR4 by butyrate (Zhang et al., 2021). It thus seems likely that differences in the cytokine profile induced by strain A2-165 compared to other strains of *F. prau* can be at least partially explained by higher butyrate production.

However, gaps remain in our understanding of *F. prau's* butyrate metabolism and its effects on patients' health, with some studies reporting inconsistent results. For example, an early study in southern India found significantly higher abundances of *F. prau* in obese children compared to non-obese children (Balamurugan et al., 2010). Similarly, Pinto et al. (2017) found increased levels of *F. prau* in T1D patients. They showed that the high abundance of the gluconeogenic enzyme phosphenolpyruvate carboxykinase in the gut proteome of their T1D cohort could be attributed to two strains of *F. prau* and hypothesized that this was due to low levels of glycolytic sources in the diets of T1D patients and a lack of acetate—necessary for butyrate production—from other bacterial sources such as *Bifidobacterium* spp. (Pinto et al., 2017). This hypothesis was supported by the finding that co-culture with strains of *Bifidobacterium* improved growth, gut colonization, and butyrate production of *F. prau.* Administration of the co-culture supernatant to mice decreased DSS-induced inflammation, providing further evidence that the beneficial properties of *F. prau* are dependent on its surrounding environment and interactions with other species (Ganesan et al., 2018; Kim et al., 2020).

Intriguingly, a 2016 study reported that samples from lean patients contained the highest count of *F. prau* genes compared to obese and T2D patients, but the lowest content of the *F. prau*–associated butyryl-CoA:acetate CoA-transferase (BUT) gene. Instead, T2D patients demonstrated the highest BUT content. This was interpreted as evidence that different phylotypes of *F. prau* produce different levels of butyrate *in vivo* and that their abundance differs from healthy to unhealthy patients (Hippe et al., 2016). It seems paradoxical that higher butyrate production by *F. prau* would be associated with obesity and T2D. The authors hypothesized that, while certain levels of butyrate production can be protective in obese patients, greatly increased production can lead to inflammation in the gut and the development of T2D. The dose-dependent effect of butyrate on epithelium permeability has long been supported by work using transepithelial electrical resistance measurements; experiments on Caco-2 cells showed protective effects at 2 mM and detrimental effects at 8 mM of butyrate (Peng et al., 2007). Similarly, a Belgian study on primary cell monolayers from ulcerative colitis patients found that although butyrate had

protective effects in control non-inflamed tissue, it actually worsened inflammation when administered together with TNF-alpha and IFN-gamma, resulting in a dramatic increase in IL-8 production (Vancamelbeke et al., 2019).

F. prau has been associated with the production of several other metabolites, including shikimic and salicylic acids, known for their antimicrobial activity, and α-ketoglutaric acid, which is involved in ammonia recycling and cell proliferation and differentiation, and known to be depleted in patients with gut dysbiosis (Miquel et al., 2015). Investigation of these other metabolites has become all the more relevant given a report that the ability of 13 different strains to decrease IL-8 levels induced by TNF-α stimulation in HT-29 cells was not correlated either to growth ratio or butyrate production (Martín et al., 2017).

Quévrain et al. (2016) identified a family of peptides from *F. prau* that were all derived from the same protein, the microbial anti-inflammatory molecule (MAM). They demonstrated that the anti-inflammatory properties of MAM arose through inactivation of the NF-κB pathway. Later experiments in models of DNBS- and DSS-induced colitis validated the effects of MAM on NF-κB *in vivo* and demonstrated its ability to inhibit the Th1 and Th17 immune responses (Breyner et al., 2017).

A more recent investigation of MAM analyzed its effects in db/db mice, which do not express leptin receptors and which demonstrated a depressed abundance of *F. prau* in the gut. When these mice were supplemented with MAM produced by *E. coli*, this protein was found to interact with ZO-1 and other tight junction proteins. Furthermore, the transfection of MAM into a cell line was able to increase ZO-1 expression and restore epithelial barrier function (Xu et al., 2019). These results suggest that MAM could have potential as an alternative treatment for pathologies involving disturbances of the gut epithelium and gut permeability.

It has thus become evident that butyrate is far from the only metabolite implicated in the immunomodulatory properties of *F. prau*. The use of this bacterium as a preventive or complementary treatment for obesity- and T2D-related inflammation of the gut could be promising. However, we first need a better understanding of the optimal dosage of bacterial units and the effects of butyrate production on host health, as well as how *F. prau* and butyrate metabolism are affected by other gut bacteria.

FINAL CONSIDERATION

Obesity is a metabolic disease caused by several factors—genetic, environmental, hormonal, and behavioral—but mainly by excess energy intake. It predisposes patients to other diseases such as hypertension and type 2 diabetes, and it is also associated with disorders of intestinal homeostasis, such as increased permeability and dysbiosis. Non-surgical treatments for obesity include changes in lifestyle, diet, and medications, all of which tend to have low adherence by the patient and are rarely effective for weight control or even for shaping intestinal health. As a major factor associated with obesity and gut health, intestinal dysbiosis may be the key to the effectiveness of weight management and the control of its associated comorbidities. For this reason, several probiotics have been tested successfully for the control of obesity, and there is intense interest in the discovery of new potential treatments.

F. prau is well known as an abundant bacterium in the natural human microbiota whose abundance is reduced in obese individuals. In addition to being a high producer of butyrate, it has anti-inflammatory effects that contribute to intestinal homeostasis. Therefore, the use of *F. prau* or its derivative products may represent a good alternative for the treatment of intestinal disorders linked with obesity and its comorbidities. However, studies on dose, forms of administration, and mechanisms of action are still necessary in order to improve our understanding of the most appropriate use of this bacterium.

AUTHOR CONTRIBUTIONS

TM contributed to design of the study and wrote the article. EB-N wrote sections of the article and drew the figure. LT, SB, and VM wrote sections in the first draft of the article. VA, PL and J-MC supervised and made critical reviews of the article. All authors approved the submitted version.

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Lyophilized Symbiotic Mitigates Mucositis Induced by 5-Fluorouracil

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Mucositis is an adverse effect of cancer chemotherapies using 5-Fluorouracil (5-FU). It is characterized by mucosal inflammation, pain, diarrhea, and weight loss. Some studies reported promising healing effects of probiotic strains, when associated with prebiotics, as adjuvant treatment of mucositis. We developed a lyophilized symbiotic product, containing skimmed milk, supplemented with whey protein isolate (WPI) and with fructooligosaccharides (FOS), and fermented by *Lactobacillus casei* BL23, *Lactiplantibacillus plantarum* B7, and *Lacticaseibacillus rhamnosus* B1. In a mice 5-FU mucositis model, this symbiotic lyophilized formulation was able to reduce weight loss and intestinal permeability. This last was determined *in vivo* by quantifying blood radioactivity after oral administration of 99mTc-DTPA. Finally, histological damages caused by 5-FU-induced mucositis were monitored. Consumption of the symbiotic formulation caused a reduced score of inflammation in the duodenum, ileum, and colon. In addition, it decreased levels of pro-inflammatory cytokines IL-1β, IL-6, IL-17, and TNF-α in the mice ileum. The symbiotic product developed in this work thus represents a promising adjuvant treatment of mucositis.

Keywords: probiotic, chemotherapy, prebiotic, immunomodulant effects, symbiotic

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INTRODUCTION

Mucositis consists of an inflammation, mainly of the small bowel, that affects individuals submitted to cancer chemotherapy treatments, such as 5-Flourouracil (5-FU) (Sonis, 2004). It includes mucosal injury, inflammation, diarrhea, and weight loss. It may lead to mucosal lesions and/or ulcerations throughout the gastrointestinal tract (Rodríguez-Caballero et al., 2012). Mucositis markers include the presence of leukocyte infiltrate in the lamina propria, degenerate enterocytes (Ciorba et al., 2016), accumulation of neutrophils and eosinophils (Antunes et al., 2016), increased degeneration of goblet cells (Stringer, 2013), as well as atrophy of villi (Chang et al., 2012).

There is presently no effective treatment for the prevention or alleviation of symptoms of mucositis. Furthermore, the use of chemotherapeutics causes severe dysbiosis (imbalance in the intestinal microbiota) which in turn worsens intestinal inflammation (van der Velden et al., 2014). In this context, development of alternative or adjuvant treatments is needed. Indeed, the use of probiotics as

promising candidates for adjuvant treatment of mucositis recently attracted attention (Carvalho RD. et al., 2017). Selected lactic acid bacteria (LAB) strains were reported as probiotics with beneficial effects mediated by different mechanisms of action and offer new perspectives for the development of adapted functional foods (Carvalho RDO. et al., 2017; Eales et al., 2017; Tang et al., 2017). Thus, studies have been carried out to evaluate the potential of such probiotic strains, associated with prebiotics, as possible symbiotic treatments of mucositis (Bastos et al., 2016).

Administration of lactobacilli strains, or of probiotic formulations, can, in pre-clinical models, alleviate experimental mucositis and prevent weight loss, diarrhea, and intestinal damages (Justino et al., 2015; Cordeiro et al., 2018; Do Carmo et al., 2019). As an example, Cordeiro and collaborators showed that the *L. casei* BL23 strain, when grown in milk supplemented with whey protein isolate, was able to mitigate inflammation in 5-FU-induced mucositis in mice (Cordeiro et al., 2018). Moreover, Galdino et al. (2018) demonstrated that Fructooligosaccharides (FOS), recognized as prebiotic, were able to reduce mucosal damages in such a model (Galdino et al., 2018). Trindade et al. (2018) further reported that the use of the symbiotic Simbioflora® reduced intestinal injury in such a model (Trindade et al., 2018). Products combining probiotics and prebiotics are called symbiotic. They may contain one or more probiotic strain(s) and one or more prebiotic compound(s) (Flesch et al., 2014). They are designed to favor synergy between the combined elements, providing the consumer with the beneficial effects of this association (Flesch et al., 2014). Several studies carried out with symbiotics highlighted effects such as reduction of pro-inflammatory cytokines (Ishikawa et al., 2005), stimulation of the immune system (Raizel et al., 2011), and reduction of intestinal infections and intestinal inflammation (Santos et al., 2015). Futher efforts focused on the quest for new strains, or consortia thereof, to be used as adjuvants in the treatment of mucositis (Picó-Monllor and Mingot-Ascencao, 2019; Shu et al., 2020). In this quest, new candidate strains *Lactiplantibacillus plantarum* B7 and *Lacticaseibacillus rhamnosus* D1 may open new perspectives. They were recently shown to prevent infections by *Salmonella enterica* serovar Typhimurium in BALB/c mice and in germ-free-mice, including clinical manifestations such as tissue damages at the level of ileum (Acurcio L. B. et al., 2017, Acurcio et al., 2017 LB.; Valente et al., 2019). This protective effect being anti-inflammatory, these strains seem good candidates to be investigated in the context of mucositis.

The aim of this work was thus to develop a symbiotic and lyophilized product, based on milk, supplemented with WPI and FOS, fermented by strains *L. casei* BL23, *L. plantarum* B7, and *L. rhamnosus* B1, which would be able to reduce the intestinal inflammation, to control the pro-inflammatory immune response, and to decrease intestinal permeability, in a murine model of mucositis induced by 5-FU.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The bacterial strain *L. casei* BL23 is part of the UMR1219 MICALIS INRA-AgroParisTech collection, Jouy-en-Josas, France. The strains *L. plantarum* B7 and *L. rhamnosus* D1 were provided by Professor Leonardo Acúrcio of Microorganisms and Ecology Laboratory of Physiology, Universidade Federal de Minas Gerais. An aliquot of the bacterial strains *L. casei* BL23, *L. plantarum* B7, and *L. rhamnosus* D1 were first inoculated (2% v/v) in MRS culture medium (deMan, Rogosa, and Sharpe) for 24 h at 37°C. Aliquots of bacterial cultures from each strain were then inoculated into 12% w/v low-fat milk medium (0.1% w/v yeast extract, 2% w/v glucose) supplemented or in the absence of whey protein isolate (WPI) 30% w/v. After growth (24 h 37°C), a 1 ml aliquot of each inoculum was removed to assess colony forming unit (CFU) count. Subsequently, 500 ml of each sample along with 500 ml of skimmed milk supplemented with WPI without the presence of bacteria (Matrix), were refrigerated and lyophilized in LH modelo 0601 (LIOMEAL—LBR Liofilização do Brasil). Posteriorly, for 5-FU-induced mucositis mice model, all three strains fermented beverages (*L. casei* BL23, *L. plantarum* B7, and *L. rhamnosus* D1) subjected to lyophilization were homogenized in a 1:1:1(g) ratio and, subsequently, added with FOS (NewNutrition®) in a sterile environment also at a ratio of 1:1(g). The product composed of three strains lyophilized in a matrix and supplemented with FOS was called Symbiotic.

Physicochemical Analyses and Bioactivity

The determination of moisture, protein, and fat content were evaluated according to what was previously described (BRASIL, 2006). To determine the moisture content, we oven-dry 5 g of a sample at 100–105°C, for 24 h. For protein content quantification and fat levels, we realized the Kjeldahl and Gerber method, respectively (Cordeiro et al., 2021). All results were expressed as g/100 g. The bioactive peptides levels were determinated evaluate the angiotensin I-converting enzyme inhibition (ACEI), antioxidant activity (DPPH), and α-amylase and α-glucosidase inhibition. For angiotensin I-converting enzyme inhibitory (ACEI) calculate we used the spectrophotometric assay, according to Konrad et al. (2014). For 2,2-diphenyl-1-picrylhydrazyl (DPPH) measurmeant, we used the radical-scavenging method previously described (Lee et al., 2016). Finally, for measurement of α-glucosidase and α-amylase inhibitory activities of lyophilized formulations we used the protocol describe by Grom et al. (2020).

Evaluation of Probiotics Properties of the Formulations in Mucosite Mice Models

Animals

Conventional BALB/c mice (female) between 6 and 8 weeks of age, obtained at Universidade Federal de Minas Gerais (UFMG–Belo Horizonte, Brazil), were used. All mice were kept in a room with temperature-controlled and standard chow diet and *ad libitum* access to water. This study was approved by the Ethics Committee on Animal Experimentation of the Federal University of Minas Gerais (379/2018).

Experimental Set-Up

For probiotic treatment, mice were gavaged daily with 500 mg (per day per animal) of dried product resuspended in PBS pH 7.4 until dissolution (500 µl for maximum final volume) for 13 days. The maximal volume given daily by gavage was set according to the good practice guide to the administration of substances (Diehl

et al., 2001). To induce the mucositis disease, on day 11, mice of inflamed groups received a single injection of 5-FU (Fauldfluor—Libbs) (300 mg/kg, intraperitoneally). An injection of saline (NaCl 0.9%) was used in control groups. After 72 h of this mucositis induction, all mice were euthanized (Carvalho RD. et al., 2017). A longitudinal abdominal incision was performed to remove the intestine for further analyses. The mice were weighed daily. BALB/c mice were divided into five groups. The non-inflamed control (naive) and inflamed control (5-FU) groups received 500 µl of PBS pH 7.4, the other three groups received the dose of 5-FU, and were gavaged with 500 mg of samples resuspended in PBS pH 7.4, i.e., the matrix control group was gavaged with the lyophilization matrix (with FOS). The *L. casei* BL23 group and the Symbiotic group were gavaged with probiotic formulations containing in addtion to WPI (lyophilization matrix) and FOS (250 mg per dose), either BL23 (10^9 CFU) or the 3 strains (10^9 CFU). The maximal volume given daily by gavage was set according to the good practice guide to the administration of substances (Diehl et al., 2001). Each group contained 18 animals.

Histopathological Analysis

The distal portion of the duodenum, jejunum, and ileum from mice was collected and prepared for histomorphological analysis. For that, tissues were immersed in 4% buffered formaldehyde solution and then the material was embedded in paraffin, and a 4-µm section of each sample was placed on a glass slide and stained with hematoxylin-eosin (HE). The histological score was done by a pathologist, using Soares et al. (2008) protocol. In this protocol, the intensity of the infiltrate of mononuclear and polymorphonuclear cells in the lamina propria of the duodenum, jejunum, and ileum, the presence of ulceration and erosion and changes in mucosal architecture were measured (Soares et al., 2008). For each parameter a classification was given according to the severity of the lesion in the tissues: absent (0), mild (1), moderate (2), and severe (3). For morphometric analysis, 10 images of the ileum of each animal were randomly captured and analyzed using ImageJ software (version 1.8.0). Additional cuts in the paraffinized samples from duodenum, jejunum, and ileum were stained by the Periodic Acid-Schiff (PAS) to determine the number of goblet cells in the tissues (Prisciandaro et al., 2011). Ten random field images of each sample were made using the 40x objective and the intact goblet cells were counted using ImageJ software (version 1.8.0) and expressed as the number of cells per high-power field (hpf) (40x, 108.2 μm^2) (Cordeiro et al., 2018).

Intestinal Permeability

To assess intestinal permeability, after 72 h of mucositis induction, a group of animals received 0.1 ml of diethylenetriaminepentaacetate acid (DTPA), labelled with 18.5 MBq of ^{99m}technetium, by gavage. Four hours later, the blood was collected, placed in appropriate tubes for radioactive determination and weighing (de Barros et al., 2018). Results were calculated as percentage of dose per g of blood, by the following equation: % dose/g blood = (cpm in g of blood/cpm dose of standard) × 100 cpm (counts of radioactivity per minute) (Galdino et al., 2018; Do Carmo et al., 2019).

Gene Expression Analysis in the Mice Ileum

Fragments of 1 cm of ileum were collected and total RNA of samples was extracted using PureLink RNA Mini Kit (Thermo Fisher Scientific). The extraction protocol was done according to the manufacturer. To digest and remove residual genomic DNA of samples we used DNase I (Invitrogen; Waltham, MA) and Turbo DNA-free Kit (Ambion; Austin, TX). RNA quality was assessed using agarose gel and NanoDrop® ND-1000 (260/230 ratio). To prepare the cDNA libraries we used the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems; Foster City, CA). Quantitative PCR (qPCR) was determined using iTaq universal SYBR green supermix (Biorad; Hercules, CA) and gene specific-primers, were selected according to Do Carmo et al. (2019), for zonula occludes 1 and 2 (*zo-1* and *zo-2*, respectively), occludin (*ocln*), claudin-1 (*cln-1*), and claudin-5 (*cln-5*). For housekeeping genes, we encoded β-actin (*actβ*) and GAPDH (*gapdh*). The amplification cycles were performed as described: 95°C for 30 s, and 40 cycles of 95°C for 15 s and 60°C for 30 s on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Results were expressed as a fold-change of expression levels, using the mean and standard deviations of target expression ($2^{-\Delta\Delta Ct}$).

Ileum Tissue Preparation for Cytokine Quantification by ELISA

Pro- and anti-inflammatory cytokines were quantified by ELISA assay. Briefly, the ileum section of were weighed and homogenized (100 mg tissue/ml buffer) in PBS containing 0.05% Tween-20 (Sigma-Aldrich, St. Louis, MO), phenylmethylsulfonyl fluoride 0.1 mM (Sigma- Aldrich, St. Louis, MO), benzethonium chloride 0.1 mM (Sigma-Aldrich, St. Louis, MO), EDTA 10 mM (Synth, São Paulo, São Paulo, Brazil), and aprotinin A 20 KIU (Sigma-Aldrich, St. Louis, MO). Suspensions were centrifuged at 3.000 g for 10 min 12 and the supernatants were collected for dosage of IL-1β, IL-6, IL- 10, IL-17, TNF-α, and INF-γ cytokine according to the R and D Systems, Inc. protocols. The absorbance was measured at 492 nm using a Microplate Reader Model 680 (BIO-RAD). Samples from six animals per group were collected for the ELISA assay, homogenized, and three technical replicates performed.

Statistical Analyses

Data were performed using one-way ANOVA or two-way ANOVA followed by the Tukey or Sidak post-test. Graphs and statistical analyzes were analized using GraphPad Prism version 9.2.0 (332) for Windows (GraphPad Software, San Diego, CA). All results were presented as the mean ± standard deviation, and $p < 0.05$ was considered as statistically significant.

RESULTS

Viability of *L. casei* BL23, *L. plantarum* B7, and *L. rhamnosus* D1 Strains Submitted to Lyophilization

The viability of strains *L. casei* BL23, *L. plantarum* B7, and *L. rhamnosus* D1, submitted to lyophilization, was measured by CFU couting after rehydration of the product. All three strains,

TABLE 1 | Proximal composition of fermented milks.

Samples	Moisture	Protein	Fat	Lactose	Ash
Matrix	70.3 ± 0.85[b]	2.26 ± 0.02[b]	2.41 ± 0.19[b]	24.99 ± 0.83[b]	0.6 ± 0.0&[b]
***L. casei* BL23**	88.2 ± 0.05[a]	3.52 ± 0.24[a]	1.81 ± 0.19[a]	5.82 ± 0.16[a]	0.65 ± 0.06[a]
***L. casei* BL23 + FOS**	88.3 ± 0.04[a]	3.53 ± 0.91[a]	1.89 ± 0.28[a]	5.57 ± 0.18[a]	0.71 ± 0.07[a]
Symbiotic	89.7 ± 0.09[a]	3.54 ± 0.43[a]	1.88 ± 0.62[a]	4.22 ± 0.92[a]	0.66 ± 0.02[a]
Symbiotic + FOS	88.4 ± 0.02[a]	3.52 ± 0.21[a]	1.82 ± 0.51[a]	5.58 ± 0.70[a]	0.68 ± 0.06[a]

**Data are expressed as the mean ± standard deviation of at least 3 replicates.*
[a–f]Different letters in the same column indicate significant differences between samples (p < 0.05).

TABLE 2 | Bioactive compounds of fermented milks.

Samples	DPPH Antioxydant	ACE Inhibition	α - amylase Inhibition	α - glucosidase Inhibition
Matrix	23.7 ± 0.32[d]	23.2 ± 1.76[e]	18.2 ± 0.31[e]	20.5 ± 0.38[e]
***L. casei* BL23**	23.1 ± 0.13[d]	37.8 ± 0.24[d]	26.7 ± 0.79[d]	32.1 ± 0.16[d]
***L. casei* BL23 + FOS**	34.1 ± 0.34[c]	43.1 ± 0.98[c]	39.2 ± 0.21[c]	49.8 ± 0.18[c]
Symbiotic	44.3 ± 0.09[b]	58.2 ± 1.10[b]	49.2 ± 0.45[b]	55.6 ± 0.92[b]
Symbiotic + FOS	55.6 ± 0.28[a]	62.1 ± 0.30[a]	62.1 ± 0.22[a]	68.9 ± 0.70[a]

**Data are expressed as the mean ± standard deviation of at least 3 replicates.*
[a–f]Different letters in the same column indicate significant differences between samples (p < 0.05). The DDPH, ACE, α - amylase and α—glucosidase was expressed in %.

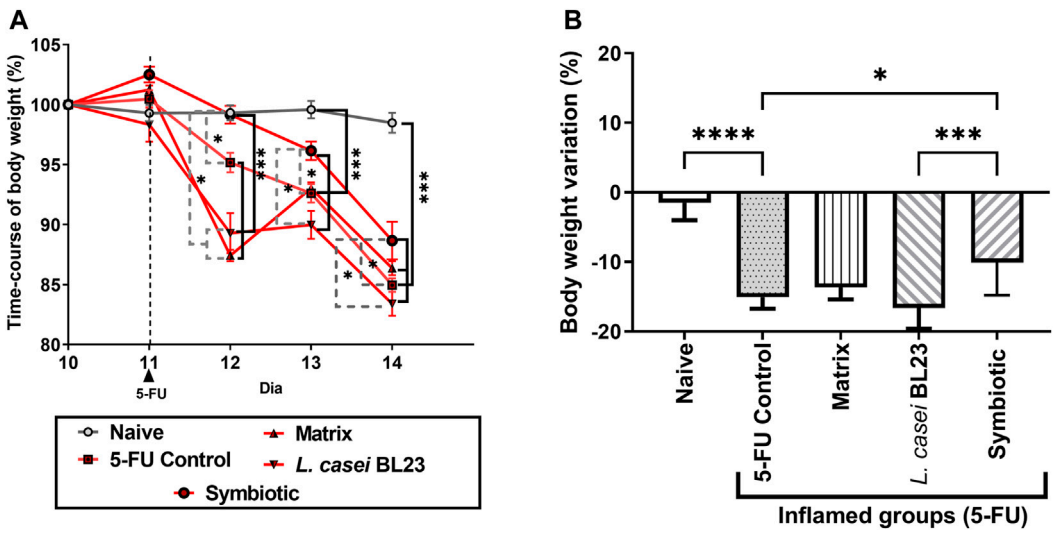

FIGURE 1 | Probiotic lyophilized formulations can interfere with weight loss in mice with 5-FU-induced mucositis. Variation in body weight of mice (in percentage) over the last 5 experimental days **(A)** of animals that were gavaged with: PBS pH 7.4 (Naive group and 5-FU inflamed control group); 12% skimmed milk supplemented with 30% WPI and Fructooligosaccharide (Matrix); a formulation containing *L. casei* BL23, a formulation containing the mix of probiotics (**Symbiotic**). **(B)** Body weight variation (in percentage) observed after the last experimental day (14th day) considering the 10th experimental day as initial weight (100%). Animals gavaged with PBS was used as experimental controls. The one-way or two-way ANOVA test, followed by Sidak or Tukey post-test was used for the multiple comparisons between groups (n = 6–9). Asterisks represent statistically significant differences as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

cultivated in the formulations with or without WPI, reached populations greater than 2×10^9 CFU/g. The population of *L. casei* BL23 was 4×10^9 CFU/g and 3×10^9 CFU/g (before and after lyophilization, respectively). That of *L. plantarum* B7 was 3.3×10^9 CFU/g and 2.3×10^9 CFU/g (before and after lyophilization, respectively). Similar results were observed with *L. rhamnosus* D1: 4.9×10^9 CFU/g and 2.5×10^9 CFU/g (before and after lyophilization, respectively).

Proximate Composition and Bioactivity Compounds

The Matrix formulation, containing WPI and FOS, was used as a control in the analysis of proximate composition and bioactivity compounds. The proximal composition of the probiotic formulations is described in **Table 1**. Analyzed parameters were moisture, proteins, lipids, lactose, and ash. It was not possible to find significant differences between the fermented

samples (*L. casei* BL23 or Symbiotic in the presence or absence of FOS). However, there was a significant difference between the Matrix control and the other formulations.

Table 2 shows the bioactive compounds in the formulations proposed in this study. Regarding the analyzed bioactives: antioxidant potential (DPPH), inhibition of the enzyme converting angiotensin ACE, inhibition of α-amylase, and inhibition of α—glucosidase, the Symbiotic lyophilized product showed the highest values with significant differences ($p < 0.05$), when compared to other lyophilized product. Moreover, there was a significant difference ($p < 0.05$) in the bioactives levels of the Symbiotic with FOS, with higher values, when compared to the Symbiotic formulation in the absence of FOS.

Symbiotic Reduces the Weight Loss in Mice with Mucositis

Figure 1 shows the time-course of mice body weight monitoring during the last five experimental days (10th to 14th day). During the pre-treatment period, prior to mucositis induction (1st to 10th day), there was no significant difference (data not shown) between the control groups (Naive and 5-FU) and the experimental groups (Matrix, *L. casei* BL23, Symbiotic). After the induction of mucositis, **Figure 1A** shows that the animals receiving the dose of 300 mg/kg of the chemotherapy 5-FU began to lose weight on the 12th experimental day, while bodyweight of control naïve mice remained constant. Moreover, all groups receiving 5-FU showed significant weight loss, when compared to the Naive group. The group of animals that received the Symbiotic treatment showed a significant difference ($p < 0.05$) in the daily variation of weight loss on the 12th and 13th experimental days. The peak of weight loss occurred on the 13th and 14th day of the experiment (**Figure 1A**). 5-FU administration, as shown in **Figure 1B**, induced weight loss in all experimental groups. However, the Symbiotic treatment significantly reduced ($p < 0.05$) the loss, compared to 5-FU (inflamed control group) and to *L. casei* BL23. We also observed a small weight loss in the Naive group without inflammation.

Symbiotic Improves Mucosal Preservation in Small Intestine of the Inflamed Mice

After euthanasia, the duodenal, jejunal, and ileal sections of the animals were collected and stained with HE and submitted to histological analysis to evaluate mucositis severity. **Figures 2A–C** show representative photos of the duodenum, jejunum, and ileum

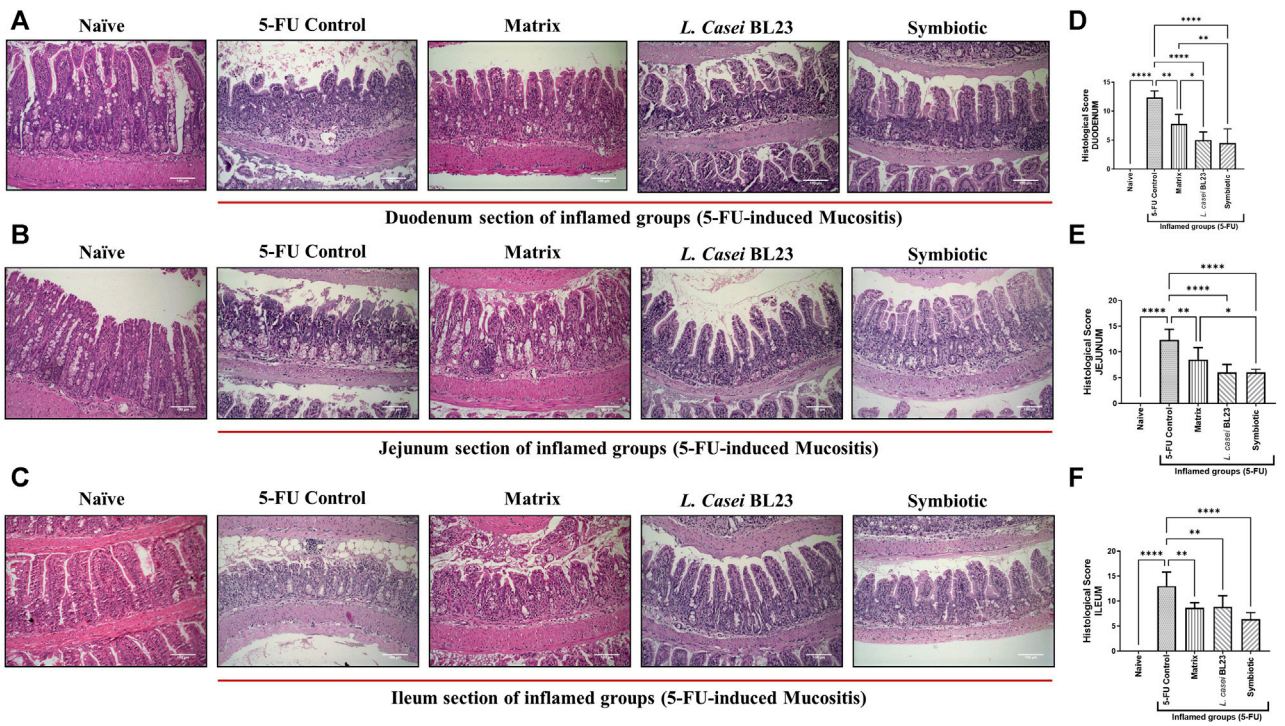

FIGURE 2 | Probiotic lyophilized formulations can reduce inflammation in the duodenal, jejunum, and ileum section of 5-FU-induced mucositis mice. **(A, B, C)** Photomicrographs of the duodenal, jejunum, and ileum of BALB/c mice, stained in HE, induced or not to mucositis by 5-FU and those that received the probiotic lyophilized formulations. The animals were gavage with: PBS pH 7.4 (Naive group and 5-FU inflamed control group); 12% skimmed milk supplemented with 30% WPI and Fructooligosaccharide (Matrix); a formulation containing *L. casei* BL23, a formulation containing the mix of probiotics (Symbiotic). The photos show the ×20 magnification. Scale bar = 100 μm **(D, E, F)** Histopathological score of the duodenal, jejunum, and ileum section. Results were expressed as means ± standard deviation (n = 6–9). One-way ANOVA and Tukey post-hoc tests were used for multiple comparison. Asterisks represent statistically significant differences as follows: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

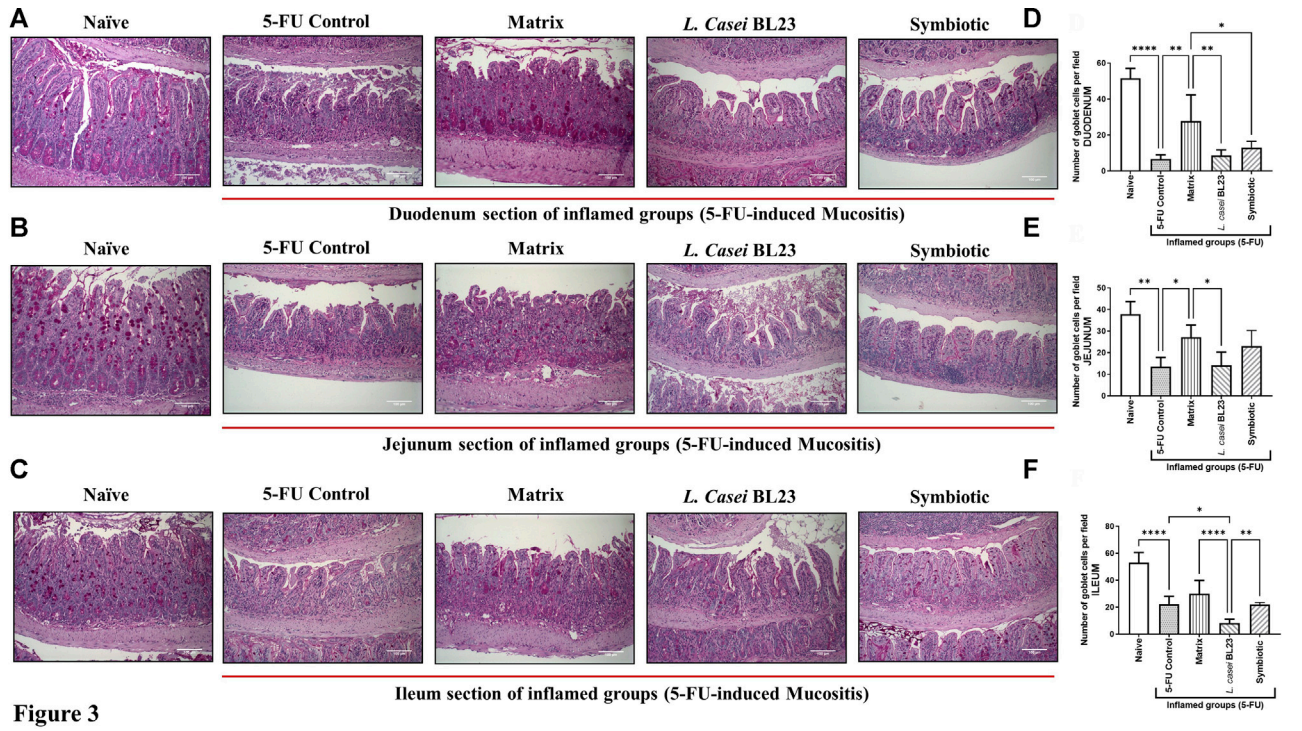

Figure 3

FIGURE 3 | Probiotic lyophilized formulations can interfere in the population of goblet cells in the duodenal, jejunum, and ileum section of 5-FU-induced mucositis mice. **(A, B, C)** Photomicrographs of the duodenal, jejunum, and ileum, stained by PAS, of BALB/c mice submitted to 5-FU-induced mucositis and those that received the probiotic lyophilized formulations. The animals were gavage with: PBS pH 7.4 (Naive group and 5-FU inflamed control group); 12% skimmed milk supplemented with 30% WPI and Fructooligosaccharide (Matrix); a formulation containing *L. casei* BL23, a formulation containing the mix of probiotics (Symbiotic). The photos show the ×20 magnification. Scale bar = 100 μm **(D, E, F)** Quantification of goblet cells in the section of the duodenal, jejunum, and ileum by field of highest magnification (×40; 108.2 μm^2). Results were expressed as means ± standard deviation (n = 6–9). One-way ANOVA and Tukey post-hoc tests were used for multiple comparison. Asterisks represent statistically significant differences as follows: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

section, respectively. In the Naive group, no morphological change in duodenum, jejunum, and ileum sections was observed according to the parameters analyzed. Sections were devoid of inflammatory infiltrate and the general architecture of the mucosa remained unchanged. In the 5-FU inflamed control group, an increase in the infiltration of inflammatory cells in the lamina propria, submucosa, and muscle layer was observed in duodenum, jejunum, and ileum section. So was an increase in the thickness of the muscle layer, as well as a drastic change in the villi, being partially or completely destroyed. Furthermore, in some animals in the 5-FU inflamed group, the presence of ulceration and erosion was observed (not shown in the images). The group that received the Matrix showed moderate preservation of the architecture and height of the villi, with a partial destruction of the crypts and the presence of a moderate (mixed) inflammatory infiltrate, reaching the mucosa and submucosa. The group that received the *L. casei* BL23 formulation showed moderate mononuclear inflammatory infiltration, moderate to intense destruction of the crypts, and a moderate preservation of the villi architecture and height. In the group that received the Symbiotic formulation, discrete destruction and reduction of villi, moderate inflammatory infiltrate with mucosal and submucosal involvement, with moderate loss of crypts, can be observed. The histopathological score of the duodenum, jejunum, and ileum sections (**Figures 2D–F**, respectively) shows a reduction in the analyzed parameters with a significant difference between the 5-FU group and the groups treated with the Matrix, *L. casei* BL23, or Symbiotic ($p < 0.01$; $p < 0.001$; $p < 0.001$, respectively). In addition, the comparative analysis identified significant differences between the group treated with the Matrix, and the group treated with Symbiotic, for scores in duodenum and jejunum section ($p < 0.05$; $p < 0.01$, respectively), with a significant reduction of the histopathological score in the latter.

As expected, the 5-FU induced mucositis in this mice model triggers substantial decrease in goblet cells number 6.66, 13.47, 22.30 goblet cell/hpf in duodenum, jejunum, and ileum section, respectively (**Figures 3A–C** for) when compared to the groups injected with 0.9% saline, 51.4, 37.9, 52.93 goblet cell/hpf in duodenum, jejunum, and ileum section, respectively. In the other hand, *L. casei* BL23 and Symbiotic treatment did not prevent the degeneration of goblet cells in the mice duodenum, jejunum, and ileum section. However, only the Matrix was able to significantly reduce the degeneration of goblet cells in the duodenum and jejunum (27.8, 27.2 goblet cell/hpf, $p < 0.05$; $p < 0.01$, respectively), not in the ileum.

Symbiotic Prevents Increase in Gut Permeability

Intestinal permeability was evaluated after oral gavage of mice with radiolabelled diethylenetriaminepentaacetate (^{99m}Tc-DTPA), followed by quantification of radioactivity in the animal´s blood. As expected, 5-FU injection significantly increased intestinal permeability ($p < 0.001$), compared to the Naive control group (**Figure 4**). There was no significant difference between the group treated with the *L. casei* BL23 and the 5-FU control group. However, animals treated with Matrix and Symbiotic exhibited significantly decreased ($p < 0.001$) intestinal permeability, compared to the 5-FU inflamed control group. Furthermore, there was no difference between the Naive, Matrix, and Symbiotic groups. Additionally, when the Matrix and Symbiotic groups were compared to the *L. casei* BL23 group, a significant reduction in intestinal permeability values was observed ($p < 0.05$).

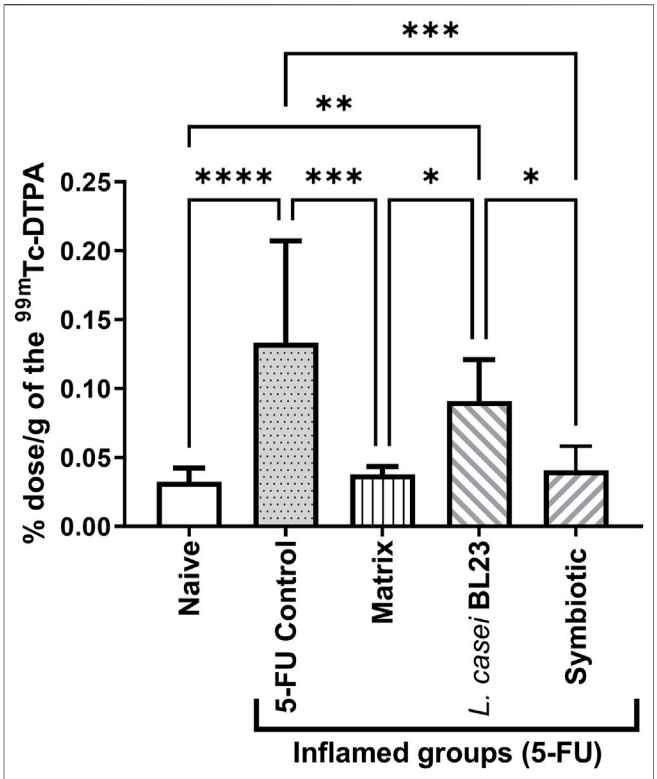

FIGURE 4 | Lyophilized probiotic formulations can decrease intestinal permeability in mice with 5-FU-induced mucositis. Intestinal permeability was measured 72 h after mucositis induction by determining Technetium-99 m radioactivity (99mTc-DTPA) in mouse blood. The animals were gavage with: PBS pH 7.4 (Naive group and 5-FU inflamed control group); 12% skimmed milk supplemented with 30% WPI and Fructooligosaccharide (Matrix); a formulation containing L. casei BL23, a formulation containing the mix of probiotics (Symbiotic). Means and standard deviations were calculated from an independent experiment for each of the 9 animals per group. Asterisks represent statistically significant differences between the strains and were indicated as follows: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ and ****$p < 0.0001$.

Symbiotic Increases Expression of Epithelial Barriers Genes

Among Naïve, 5-FU (inflamed control group), Matrix, and *L. casei* BL23 groups, no difference was found between groups in the expression of genes *zo-1*, *zo-2*, *claudin-1,* and *occluding* (**Figure 5**). However, the Symbiotic treatment was able to significantly increase the expression of the genes zo-1 ($p < 0.01$), occludin ($p < 0.05$), and claudin-1 ($p < 0.05$), when compared to the 5-FU inflamed control groups. It is noteworthy that expression of the ZO-1 and Occludin genes in the Symbiotic group was significantly higher, when compared to Matrix and *L. casei* BL23 groups, respectively, *zo-1* ($p < 0.01$; $p < 0.05$), *ocludin* ($p < 0.05$; $p < 0.05$).

Symbiotic Modulates Anti-Inflammatory Cytokines in Mice Ileum

As shown in **Figure 6**, the cytokines IL-1β, IL-6, IL-17, and TNF-α were significantly enhanced ($p < 0.001$; $p < 0.05$; $p < 0.0001$; $p < 0.001$, respectively) in the ileum of animals administered with 5-FU (inflamed control group), when compared to the Naïve control group. However, Symbiotic treatment was able to reduce significantly cytokines levels of IL-1β, IL-6, IL-17, TNF-α ($p < 0.05$; $p < 0.0001$; $p < 0.0001$; $p < 0.001$, respectively) compared to the 5-FU control group. In addition, the Symbiotic group reduced significantly ($p < 0.05$) the levels of IL-1β, when compared to the Matrix group. The Matrix and *L. casei* BL23 treatments were also able to reduce significantly cytokines levels of IL-6, IL-17, and TNF-α, compared to the 5-FU group. No difference was found in cytokine levels of INF-γ. Additionally, only *L. casei* BL23 was able to increase Il-10 levels, compared to the 5-FU inflamed control group ($p < 0.05$).

DISCUSSION

Mucositis is characterized by inflammation and by cell loss at the level of the epithelial barrier of the digestive tract. This leads to mucosal lesions and/or ulcerations throughout the TGI, i.e., from the mouth to the anus (Soares et al., 2008). Treatments aimed at controlling such side effects of cancer chemotherapy are lacking. We thus proposed to use probiotic bacterial strains, either in suitable culture media (Do Carmo et al., 2019) or in dairy matrices supplemented with whey protein (Cordeiro et al., 2018). We obtained promising results against adverse effects caused by chemotherapeutics, precisely in 5-FU-induced mucositis. Furthermore, Galdindo and collaborators used a FOS prebiotic to attenuate the effects of 5-FU-induced mucositis and obtained promising results (Galdino et al., 2018). Therefore, the present work aimed to develop a symbiotic product from milk, fermented by strains of *Lactobacillus casei* BL23, *Lactiplantibacillus plantarum* B7, and *Lacticaseibacillus rhamnosus* D1, supplemented with WPI, added with FOS, and subsequently lyophilized, to evaluate its therapeutic effects in a murine model of 5-FU-induced mucositis.

Initially, bioactive compounds analyses showed that the Symbiotic (supplemented with FOS) has high levels of the DPPH, ACE, a-amylase, and a-glucosidase inhibitor bioactive

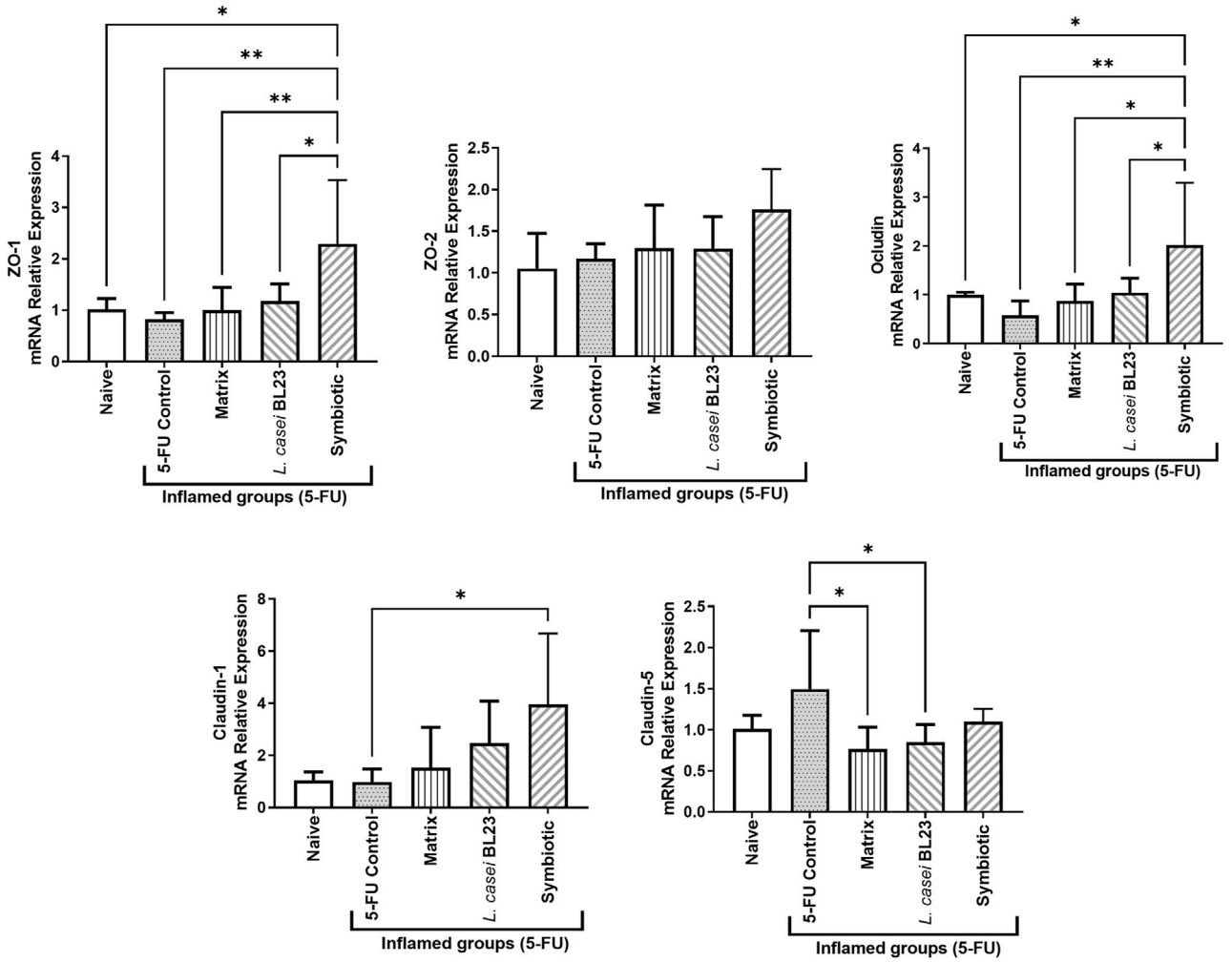

FIGURE 5 | Probiotic lyophilized formulations can modulate gene expression of epithelial barrier genes in the ileum section of mice with 5-FU-induced mucositis. Gene for zonula occludes 1 and 2 (zo-1 and zo-2, respectively), occludin (ocln), and claudin-1 (cln-1) were measured by RT-qPCR. The animals were gavage with: PBS pH 7.4 (Naive group and 5-FU inflamed control group); 12% skimmed milk supplemented with 30% WPI and Fructooligosaccharide (Matrix); a formulation containing *L. casei* BL23, a formulation containing the mix of probiotics (Symbiotic). Means and standard deviations are calculated from 3 animals per group, from 2 independent repetitions and each quantification was performed in duplicate (technical duplicate). Asterisks represent statistically significant differences between strains and were indicated as follows: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ and $^{****}p < 0.0001$.

compounds. These inhibitor peptides have beneficial effects in other intestinal inflammations such as diabetes, hypertension, obesity, and inflammatory bowel disease (Takiishi et al., 2017; Salinas et al., 2021). It is worth emphasizing the role of the antioxidant activity, which is: protective against histological damage, apoptotic changes, and pro-inflammatory cytokines, mainly caused by chemotherapeutic agents. Given the previous selection of bacterial strains due to their probiotic potential in infection *in vivo* models and mucositis (Acurcio L. B. et al., 2017, Acurcio et al., 2017 LB.; Cordeiro et al., 2018; Valente et al., 2019), and subsequent characterization of the lyophilized fermented product and supplemented with FOS, we decided to investigate its therapeutic potential in a murine model of mucositis induced by 5-FU chemotherapy.

It is known that the application of 5-Fluorouracil in mice leads to a significant weight loss, when compared to non-inflamed animals (Chang et al., 2012). As seen in previous studies, 5-FU-induced mucositis in BALB/c mice triggers a drastic reduction in weight, pasty stools with the presence of blood, as a result of substantial changes in the architecture, and destruction of the intestinal mucosa, resulting in intense inflammatory process (Carvalho RD. et al., 2017; Cordeiro et al., 2018). This also favors a change in intestinal permeability (Galdino et al., 2018; Do Carmo et al., 2019). The probiotic VSL#3 was tested in a murine model of mucositis (Bowen et al., 2007). Hence, this product, which contains several bacterial strains (one strain of *Streptococcus thermophilus*, four *Lactobacillus* spp., and three *Bifidobacterium* spp.), was effective in reducing weight loss in a mucositis model induced by irinotecan. Since mucositis affects the entire gastrointestinal tract, we decided to extend the histological analysis to include the duodenum and jejunum

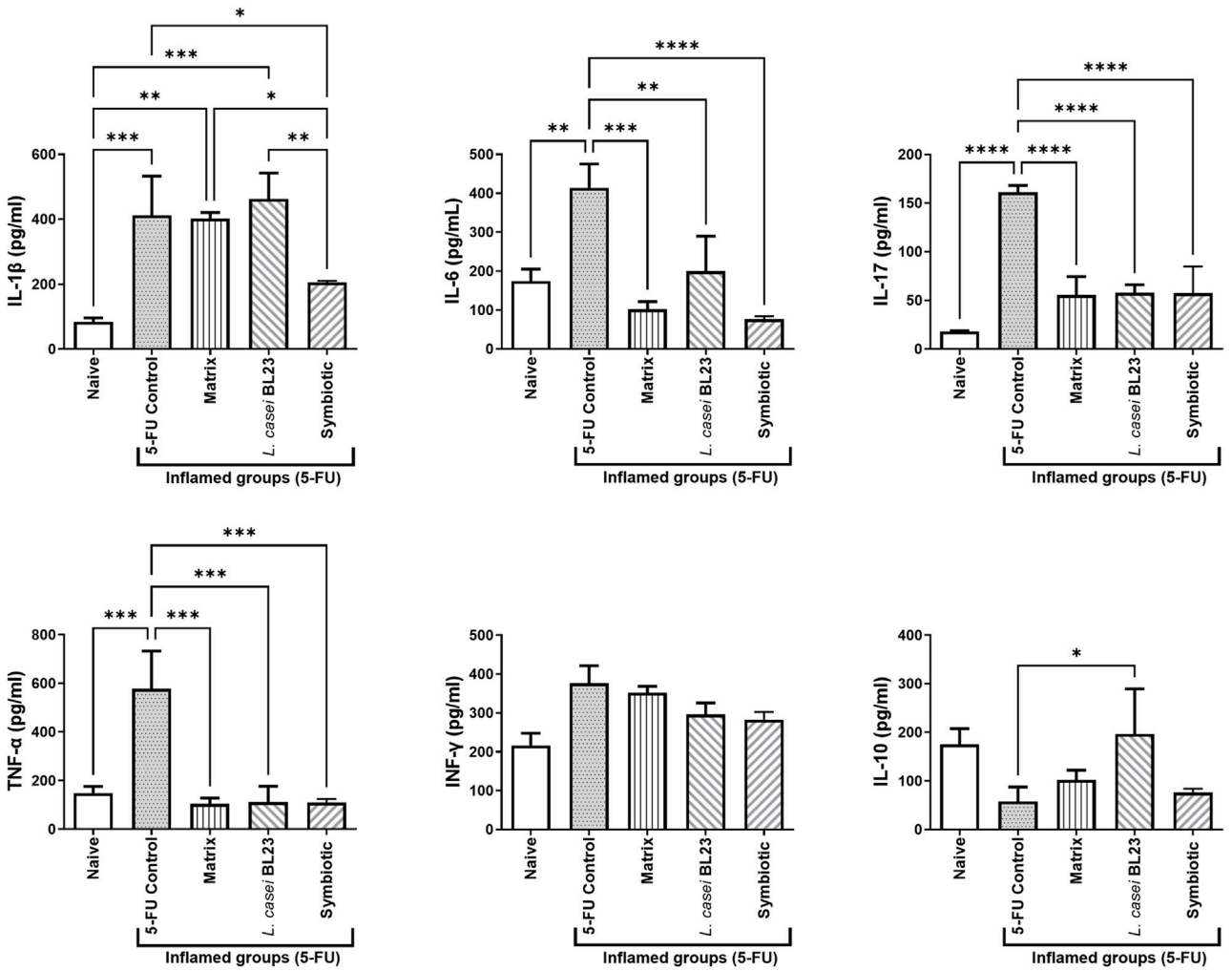

FIGURE 6 | Probiotic lyophilized formulations can modulate cytokine production in the ileum section of mice with 5-FU-induced mucositis. Cytokine levels (A IL-1β, IL-6, IL- 10, IL-17, TNF-α, and INF-γ ratio were determined in mouse ileum tissue supernatant by ELISA. The animals were gavaged with: PBS pH 7.4 (Naive group and 5-FU inflamed control group); 12% skimmed milk supplemented with 30% WPI and Fructooligosaccharide (Matrix); a formulation containing L. casei BL23, a formulation containing the mix of probiotics (Symbiotic). Means and standard deviations are calculated from 3 animals per group, from 3 independent repetitions, and each quantification was performed in triplicate (technical triplicates). Asterisks represent statistically significant differences between strains and were indicated as follows: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ and $^{****}p < 0.0001$.

section, together with the ileum section, described in the literature as the site intensely affected by 5-FU action (Touchefeu et al., 2014). In the 5-FU control group, alteration and destruction of the mucosal architecture, as well as extensive inflammation, was observed in the duodenum and jejunum. In the histological analysis of the duodenum, jejunum, and ileum, it was observed that all formulations were able to reduce inflammation in the intestinal mucosa. Interestingly, the Matrix, containing WPI and FOS, was sufficient to reduce inflammation. This result is in agreement with previous studies using WPI and FOS (Cordeiro et al., 2018; Galdino et al., 2018), which indicated significant protection. Other authors suggested that some of the amino acids present in WPI, such as cysteine and glutamate, are used to produce glutathione (Moslehi et al., 2014). This is responsible for providing the main intracellular defense against oxidative stresses, which occurs in severe inflammation such as mucositis, a fact that makes WPI a potential anti-inflammatory compound (Shiby and Mishra, 2013). Futhermore, the results found here using our Symbiotic are similar to those found in other works. For example, Yeung et al., using *Lactobacillus acidophilus* and *Bifidobacterium bifidum* strains, significantly reduced mucosal damage caused by 5-FU-induced mucositis in a murine model (Yeung et al., 2015). In the work of Trindade et al., the administration of the symbiotic Simbioflora®, which contains four probiotic strains plus FOS, also reduced damages in the same animal model (Trindade et al., 2018). We suggest that the synergy provided by the interaction of the *Lactobacillus* three strains, with previously tested anti-inflammatory potential, plus FOS, is

responsible for improving the response to inflammation. However, more studies will be needed to understand the synergistic mechanisms, the specific action of each of the strains, and the mechanism responsible for mitigation of inflammation caused by chemotherapy.

The impact of mucositis, using chemotherapeutics, expands throughout the intestinal barrier, also affecting the production of goblet cells (Carvalho RDO. et al., 2017). The group that received the lyophilization matrix, interestingly, showed a significant preservation of these cells. Perhaps, the matrix components that were not metabolized by the strains helped in the preservation of goblet cells. A hypothesis for this result, as observed by Cordeiro et al. (2018), would be the availability of amino acids via WPI, mainly threonine, cysteine, and serine for the synthesis of this mucus (Faure et al., 2006) and also the presence of FOS action, increasing the population of Bifidobacteria, which would modulate mucus production.

Mucositis alters the epithelial integrity of the gastrointestinal tract. As a result, the intestinal permeability is affected (Cinausero et al., 2017), allowing translocation of harmful and toxic substances produced by pathobionts bacteria. This may in turn allow their passage from the intestinal lumen to the blood circulation, causing unwanted systemic effects that can lead to death (Fine et al., 2020). Accordingly, 5-FU led here to increased intestinal permeability of animals in the inflamed, when compared to the Naive group. Consumption of *L. casei* BL23 failed to prevent this increase, while preserving the architecture and reducing the inflammation in the small intestine. However, the Matrix and Symbiotic both significantly reduced the intestinal permeability of mucositis mice. In the work of Galdino et al., FOS administration in 5-FU mucositis mice led to similar results (Galdino et al., 2018). In the work of Antunes et al. (2016), the use of the amino acid L-arginine, present in WPI in large quantities, reduced damages to the mucosa and intestinal permeability of animals in a murine model of mucositis. However, synergy with the strains contained here in the Symbiotic promoted an increase in the expression of genes involved in the intestinal epithelial barrier (ZO-1, Ocludin, and Claudin-1). This may explain how this formulation was able to reduce intestinal permeability.

In response to the administration of the chemotherapeutic agent 5-FU, mediators of the inflammatory response are activated, including the transcription factor NF-kb. Its activation leads to the production of pro-inflammatory cytokines, such as TNF-α, IL1-β, and IL-6 and IL-17 (Chang et al., 2012). These inflammatory markers play a central role in mucositis and are released in the inflammatory phase (Lopez-Castejon and Brough, 2011). The symbiotic was able to reduce the level of pro-inflammatory cytokines, which allows us to hypothesize that the therapeutic action of this formulation in the 5-FU-induced mucositis model is mediated by the inhibition of the pro-inflammatory response. This modulatory effect may be favored by metabolites (SCFA, bacteriocins, and neurotransmitters such as GABA) as a result of fermentation by the three strains. This would explain the difference between the Matrix and *L. casei* BL23. However, further experiments will be necessary to fully explain the mechanisms responsible for the healing effect observed here and to identify the different anti-inflammatory effectors produced by these strains.

CONCLUSION

In conclusion, we have demonstrated that the lyophilized Symbiotic formulation, containing WPI, FOS, and fermented by *Lactobacillus casei* BL23, *Lactiplantibacillus plantarum* B7 and *Lacticaseibacillus rhamnosus* B1, has anti-inflammatory potential in 5-FU-induced mucositis, reducing animal weight loss, intestinal permeability, modulating genes implicated in the intestinal epithelial barrier, controlling pro-inflammatory cytokine levels, and reducing mucosal damage caused by chemotherapy. This work opens new perspectives for the development of functional symbiotic products for target populations, in the context of mucositis, based on smart selection of matrices and bacterial consortia.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee on Animal Experimentation of the Federal University of Minas Gerais (379/2018).

AUTHOR CONTRIBUTIONS

V.A. and F.C. conceived and designed the experiments. L.A. provided strains. J.A. and A.C. performed and analyzed ELISA assays. B.S., A.G., M.Q., B.C., E.O., S.H., G.B., and A.G-G. were major contributors to animal experimentation. J.G., R.S., R.R., M.S., M.F., E.E., and A.G-G. performed centesimal and mineral composition. E.F. performed, analyzed, and interpreted the histological analysis from colon slides. K.V., S.F., and V.C. performed permeability assays. T.D. and G.C. perfomed RT-PCR analyses. V.A. and F.C. wrote the original draft. G.J. and Y.L. gave scientific advice and participated in the writing of the manuscript. All authors contributed to data interpretation, drafting the manuscript, critically revising the manuscript, and approving its final version.

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SlpB Protein Enhances the Probiotic Potential of *L. lactis* NCDO 2118 in Colitis Mice Model

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Bacteria used in the production of fermented food products have been investigated for their potential role as modulators of inflammation in gastrointestinal tract disorders such as inflammatory bowel diseases (IBD) that cause irreversible changes in the structure and function of gut tissues. Ulcerative colitis (UC) is the most prevalent IBD in the population of Western countries, and it is marked by symptoms such as weight loss, rectal bleeding, diarrhea, shortening of the colon, and destruction of the epithelial layer. The strain *Propionibacterium freudenreichii* CIRM-BIA 129 recently revealed promising immunomodulatory properties that greatly rely on surface-layer proteins (Slp), notably SlpB. We, thus, cloned the sequence encoding the SlpB protein into the pXIES-SEC expression and secretion vector, and expressed the propionibacterial protein in the lactic acid bacterium *Lactococcus lactis* NCDO 2118. The probiotic potential of *L. lactis* NCDO 2118 harboring pXIES-SEC:*slpB* (*L. lactis*-SlpB) was evaluated in a UC-mice model induced by Dextran Sulfate Sodium (DSS). During colitis induction, mice receiving *L. lactis*-SlpB exhibited reduced severity of colitis, with lower weight loss, lower disease activity index, limited shortening of the colon length, and reduced histopathological score, with significant differences, compared with the DSS group and the group treated with *L. lactis* NCDO 2118 wild-type strain. Moreover, *L. lactis*-SlpB administration increased the expression of genes encoding tight junction proteins *zo-1*, *cln*-1, *cln-5*, *ocln*, and *muc-2* in the colon, increased IL-10 and TGF-β, and decreased IL-17, TNF-α, and IL-12 cytokines in the colon. Therefore, this work demonstrates that SlpB recombinant protein is able to increase the probiotic potential of the *L. lactis* strain to alleviate DSS-induced colitis in mice. This opens perspectives for the development of new approaches to enhance the probiotic potential of strains by the addition of SlpB protein.

Keywords: SlpB, propionibacterium, colitis, *Lactococcus lactis*, inflammatory bowel disease

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INTRODUCTION

Propionibacterium freudenreichii (*Pf*) is a dairy propionic acid bacterium (PAB) that has gained prominence as a potential probiotic, after studies have shown primitive characteristics, such as the production of short-chain fatty acids and conjugated linoleic acid, in addition to producing vitamin 12 at an industrial scale (Thierry et al., 2011; Deptula et al., 2017). *Pf* has been listed in the

qualified presumption of safety (QPS) list by the European food safety authority and has a GRAS (Generally Recognized As Safe) status for its use in cheese. The immunomodulatory properties of some *Pf* strains have already been clearly demonstrated in inflammatory bowel disease (IBD) mice models (Foligne et al., 2010; Carvalho et al., 2017; Ma et al., 2020; Rabah et al., 2020) and in the mucositis model (Cordeiro et al., 2018; Do Carmo et al., 2020). The probiotic properties of *Pf* are directly linked to the presence of surface proteins, the S-layer proteins (Slp), as shown by the study by Do Carmo et al. (2017) and Do Carmo et al. (2018). Mutation of the gene encoding SlpB, a surface protein present at the surface of the probiotic strain *P. freudenreichii* CIRM-BIA 129 (*Pf* 129), drastically alters its immunomodulatory effects *in vitro* and *in vivo*, its adhesion to HT-29, its physicochemical properties, its ability to survive stress, and its surface and whole-cell proteome. Moreover, the purified *Pf* 129 SlpB protein was able to increase IL-10 gene expression in HT-29 cells. Furthermore, it is very important to know whether the immunomodulatory effects of the *Pf* 129 SlpB protein can be observed in other organisms, such as lactic acid bacteria.

Probiotic potential of the lactic acid bacterium (LAB) *Lactococcus lactis* has widely been explored. Notably, recent studies on the *L. lactis* subsp. *lactis* strain NCDO 2118 pointed out its potential to control intestinal inflammation in a mouse model (Carvalho et al., 2017). Precisely, *L. lactis* NCDO 2118 is amenable to transformation, and it has been used for the production and secretion of heterologous proteins in a *L. lactis* species that reportedly secretes a small number of homologous proteins (Nouaille et al., 2003). Miyoshi and collaborators (2004) developed a versatile plasmidic expression system inducible by xylose (xylose-inducible expression system—XIES). XIES plasmid (pXIES) can address the recombinant protein to the cytoplasm (p*XIES-CYT*) or to the extracellular medium (p*XIES-SEC*) (Miyoshi et al., 2004). Gomes-Santos et al. (2017) explored the potential of *L. lactis* strain NCDO 2118 secreting the *Mycobacterium leprae* heat-shock protein HSP65 (p*XIES-SEC*:*hsp*65) and obtained promising results in mitigating experimental colitis in mice model.

Probiotics, such as *P. freudenreichii* CIRM-BIA 129 and *L. lactis* NCDO 2118, have been tested as adjuvants in the treatment of colitis in animal models (Gomes-Santos et al., 2017; Ma et al., 2020; Rabah et al., 2020; Cordeiro et al., 2021). IBDs induce pathological signs and symptoms such as weight loss, rectal bleeding, diarrhea, shortening of the colon, and destruction of the epithelial layer. In the colon mucosa of patients affected by ulcerative colitis (UC), the presence of an inflammatory infiltrate composed of neutrophils and eosinophils are described. Furthermore, destruction of the epithelial barrier and the mucin layer leads to the exposure to antigens or pathobionts present in the intestinal lumen, exacerbating the pro-inflammatory response (Kushkevych and Monika, 2021). IBD etiology is being explored to unravel the mechanisms responsible for this pathology. Beyond the evidence of genetic susceptibility, the intestinal microbiota alterations (or dysbiosis), causing an exacerbated immune response in the host, can affect and aggravate IBD symptoms. An experimental approach proposed for the study of IBDs is a mice model of colitis induced by Dextran Sulfate Sodium (DSS). DSS-induced colitis model is able to mimic and reproduce IBD pathology routinely observed in human UC, body weight reduction, diarrhea, bloody feces, decreased colon length, mucosal injury, impaired mucus epithelial barrier function, and proinflammatory immune response (Wirtz et al., 2017).

In this work, we explore the therapeutic role of *Pf* 129 SlpB protein in the modulation of intestinal inflammations induced by chemical substances. In this aim, we use the *L. lactis* NCDO2118 harboring pXYSEC:*slpB*, to evaluate its effects in the DSS-colitis mice model.

MATERIALS AND METHODS

Strains and Cloning Procedure

Lactococcus lactis NCDO 2118 wild-type (*L. lactis* WT) strain was grown at 30°C in M17 medium (Difco) containing 0.5% glucose (GM17), without agitation, or in the same medium solidified with 1.5% agar for 18 h. The nucleotide sequence encoding the SlpB surface protein from *Propionibacterium freudenreichii* CIRM-BIA 129 (*Pf* 129) was obtained from the database of the National Center for Biotechnology Information (NCBI), deposited under accession number CDP48273.1 (CDS 5503..7173). The sequence was optimized for expression in *Lactococcus lactis* NCDO2118 bacteria in the OptimumGeneTM program (GenScript Corporation) and synthesized by GenScript Corporation (Piscataway, NJ, USA) and cloned into pUC57 vector. The optimized SlpB protein sequence was synthesized with the restriction sites *Nsi*I-3′ and *Eco*RI-5 to cloning into the plasmid pXYSEC (chloramphenicol resistance). The NsiI/EcoRI digested and purified SlpB ORF and pXY:SEC fragments were ligated by T4 DNA ligase (Invitrogen) to obtain the pXYSEC:*slpB* plasmid, which was established by transformation in *E. coli* Top10 and selected with 10 μg/ml of chloramphenicol (Cm) in Luria Bertani Agar (Miyoshi et al., 2004). Routinely, the *primers* SlpB-Forward 5′-GATCCCCCG TCTGAACGAACTT-3′ and SlpB-Reverse 5′-CGACATCAT TGAACATGCTGAAGAGC-3′ were used for plasmid construction verification by PCR and agarose gel, and also for sequencing (PCR product size: 1,816 bp). Then, the optimized gene sequence for SlpB was subcloned into the pXYSEC plasmid and competent *L. lactis* NCDO 2118 bacteria was transformed by electroporation as previously described by Langella et al. (1993), and grown at 30°C in M17 medium (Difco) containing 0.5% glucose (GM17) without agitation containing 10 μg/ml of chloramphenicol. To confirm the final construction of pXYSEC:*slpB*, a DNA sequencing was performed by fluorochrome-labeled dideoxynucleotides method (BigDye Terminator v3.1 Cycle Sequencing, Applied Biosystems, USA). Recombinant *L. lactis* NCDO2118 strain was grown in Difco M17 broth, supplemented with either 0.5% glucose (GM17) or 1% xylose (XM17) and chloramphenicol (10 μg/ml) at 30°C without agitation. On the first day, single colonies of recombinant *L. lactis* NCDO2118 harboring pXYSEC:*slpB* (*L. lactis*-SlpB) were cultured in 5 ml of GM17. On the second day, the overnight

culture was diluted 1:10,000 in XM17 to induce the expression of the *slpB* gene ORF. Proteins sample preparation from *L. lactis* wild type and recombinant *L. lactis*-SlpB cultures was performed as previously described (Miyoshi et al., 2004). To verify protein production, a Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting was done as previously described by Do Carmo et al., 2017.

Animals

Conventional female C57BL/6 mice of 8 weeks of age, obtained at the Universidade Federal de Minas Gerais (UFMG, Belo Horizonte, Brazil), were used in this work. They were housed in plastic cages in a room with controlled temperature (18°C–23°C), light cycle 14 h light/10 h dark, relative humidity (40%–60%), and *ad-libitum* access to food and water. All experimental procedures realized in this work were approved by the Ethics Committee on Animal Experimentation of the Universidade Federal de Minas Gerais (CEUA-UFMG, Brazil) by the protocol no. 148/2020.

Experimental Design and Dextran Sulfate Sodium-Induced Colitis

L. lactis NCDO2118 WT (*L. lactis*) and *L. lactis* NCDO2118 pXYSEC: *slpB* (*L. lactis*-SlpB) strains were prepared daily for the animals, using intragastric gavage as a form of administration. Both strains were grown in M17 medium (Difco) added with glucose (0.5%), for the wild-type strain, and xylose (1%) + 10 μg/ml of Cm, for the *L. lactis*-SlpB strain. Bacteria cultures (*L. lactis* and *L. lactis*-SlpB), were incubated for 24 h at 30°C, and 1 ml of each culture was centrifuged at 3,500 rpm for 10 min and washed with PBS pH 7.4 twice to remove the antibiotic. Thus, each bacterial pellet corresponds to a daily dose with 5×10^9 CFU/per dose of bacteria, which was then resuspended in 100 μl of PBS pH 7.4.

The mice were divided randomly into four main groups, each containing six animals per group (**Supplementary Figure S1**). Group 1 represented a healthy control group that received only water for drinking (control group). The mice from groups 2–4 (experimental groups) received DSS (36–50 kDa, MP Biomedicals, CAT 260110, LOT Q5756), as the only drinking source, prepared to a concentration of 1.7% in filtered drinking water and provided to the animals daily, during 7 days, according to the acute colitis model previously described (Wirtz et al., 2017). Animals from group 2 received only DSS solution (group DSS) and no treatment. Mice from groups 3 and 4 received the bacteria dose during the 7 days by gavage (all experimental days), together with DSS in drinking water. Precisely, mice from group 3 received intragastric doses (100 μl containing 5×10^9 CFU) of *L. lactis* NCDO 2118 WT (group DSS + NCDO 2118 WT), and animals in group 4 received (100 μl containing 5×10^9 CFU) of *L. lactis* NCDO 2118 pXYSEC: *slpB* (group DSS + NCDO2118 pXYSEC:*slpB*). The mice were euthanized on the seventh day. All *in vivo* experiments were done in biological triplicate.

Assessment of Colitis Severity

During all experimental days, the water, food intake, and mice body weight were recorded daily. On the last experimental day, the disease activity index (DAI) was determined, as described by Cooper et al. (1993), attributing a score of the three major colitis clinical signs: weight loss, intensity of diarrhea, and presence of rectal bleeding.

A longitudinal abdominal incision was performed in all mice to access the intestine and colon, and then to be used in future analyses. The colon length of each mouse (measured from the cecum to rectum) were used to indicate the mean of each experimental group (cm). The colon distal part was collected, washed with PBS, and stored in segment rolls for histomorphological analysis. These rolls were immersed in formaldehyde solution (4%, v/v) for tissue fixation and, after that, they were embedded in paraffin. A section (4 μm) was placed on a glass slide and stained with Hematoxylin-Eosin (HE) (Marchal Bressenot et al., 2015). The sections were photographed (×20 magnification objective) using a digital camera (Spot Insight Color) coupled to an optical microscope (Olympus, BX-41, Japan). The histological inflammation score was determined by a pathologist using the score previously described by Wirtz et al. (2017). Consider: tissue damage (0: none; 1: isolated focal epithelial damage; 2: mucosal erosions and ulcerations; 3: extensive damage deep into the bowel wall) and lamina propria inflammatory cell infiltration (0: infrequent; 1: increased, some neutrophils; 2: the submucosal presence of inflammatory cell clusters; 3: transmural cell infiltrations). The total score ranging from 0 (no changes) to 6 (widespread cellular infiltrations and extensive tissue damage) were obtained by the sum of these two sub-scores (tissue damage and lamina propria inflammatory cell infiltration). Other cuts of the paraffinized colon samples were produced and stained by the periodic acid-Schiff (PAS) (Prisciandaro et al., 2011) in order to count the mucus-producing goblet cells. Ten random field images from each sample were made using the ×40 objective, and then, using ImageJ software (version 1.8.0) the intact goblet cells were counted. The total number of goblet cells was expressed as the number of cells per high-power field (HPF) (×40, 108.2 μm^2).

Colonic Activity of Myeloperoxidase and the Eosinophil Peroxidase

Neutrophil infiltration levels in the colon tissue were assessed by measurement of myeloperoxidase activity (MPO), as previously described by Porto et al., 2019. For MPO quantification, a piece of colon tissue (100 mg) was homogenized proportionally in 1.9 ml/100 mg of PBS and centrifuged at 10,000 × *g* for 10 min. The pellet formed was lysed and centrifuged again. The pellet formed was resuspended proportionally in 1.9 ml/100 mg of 0.5% HTAB (hexadecyltrimethylammonium bromide) diluted in PBS. The suspension was submitted to freeze–thaw cycle (3x) using liquid nitrogen and then, centrifuged at 12,000 × *g* at 4°C, for 10 min. In order to perform the enzymatic assay, we added an equal amount of substrate (1.5 mM L^{-1} of o-phenylenediamine and 6.6 mM L^{-1} of H_2O_2 in 0.075 mM L^{-1} of Tris–HCl pH 8.0) to the supernatant. To stop the enzymatic reaction, 50 μl of 1 M H_2SO_4 was added. The absorbance was read in a spectrophotometer (Spectramax M3, Molecular Devices, LLC, Sunnyvale, CA, USA), at 492 nm.

The extent of eosinophil infiltration into the tissues was assessed by measuring eosinophil peroxidase (EPO) activity, as previously described by Vieira et al. (2009). For EPO quantification, a piece of colon tissue (100 mg) was homogenized proportionally in 1.9 ml/100 mg of PBS and centrifuged at 10,000 × *g* for 10 min 4°C. The precipitate was subjected to hypotonic lysis, where 0.9 ml of a solution containing 0.2% NaCl was added prior to the addition of an equal volume of solution containing 1.6% NaCl and 5% glucose. The samples were again homogenized and centrifuged (10,000 × *g*, at 4°C, for 10 min). The supernatant was discarded, and the pellet was resuspended in 1.9 ml of 0.5% HTAB (hexadecyltrimethylammonium bromide) diluted in PBS. After three cycles of freeze–thaw in liquid nitrogen, the samples were centrifuged at 4°C, 10,000 g for 10 min. To test the enzyme activity, the obtained supernatant was mixed with a substrate (1:1) containing 1.5 mmol/L of o-phenylenediamine, 6.6 mmol/L of H_2O_2, and 0.075 mmol/L of Tris-HCl pH 8. After 30 min the reaction was stopped with 50 µl of 1 M H_2SO_4. The absorption was measured in a spectrophotometer (Spectramax M3, Molecular Devices, LLC, Sunnyvale, CA, United States) at 492 nm.

Measurement of Secretory Immunoglobulin A

The secretory immunoglobulin A (sIgA) of the intestinal lavage was determined by ELISA, according to Cordeiro et al., 2021. For the quantification of the samples was used a 96 well-plates (Nunc-Immuno Plates, MaxiSorp) coated with anti-IgA antibodies (Southern Biotechnology, Birmingham, AL, United States) and incubated overnight. After the incubation, the plates were washed in saline-Tween (saline with 0.05% of Tween-20—SIGMA Chemical Co.) and blocked with 200 µl of PBS-casein (0.05%) for 1 h, at room temperature. After that, the intestinal lavage contents were added, and the plate was serially diluted (1:100) and incubated at room temperature for 1 h. Plates were washed with saline-Tween and then, biotin-conjugated anti-mouse IgA antibodies were added (Southern Biotechnology) (1: 10,000 in PBS-casein). Plates were incubated for 1 h at 37°C and then, biotinylated monoclonal antibodies anti-IgA (BD Bioscience) were added and incubated for 1 h at room temperature. Following this, peroxidase-labeled streptavidin (Southern Biotechnology) was added. Plates were washed in saline-Tween and incubated again with 100 µl of orthophenylenediamine (OPD) (Sigma, St. Louis, MO, USA) and H_2O_2 (0.04%), for 1 h, at room temperature. To stop the reaction, 20 µl/well of 2N H_2SO_4 was added. Absorbance reading was performed on Bio-Rad Model 450 Microplate Reader, at 492 nm. The results of total sIgA were measured, according to the standard curve, in a concentration of sIgA (ng) per ml of intestinal fluid.

Colonic Gene Expression Analysis

In order to obtain the quantitative gene expression in colon fragments, the methodology was carried out according to Do Carmo et al. (2020). Fragments of 1 cm of the colon were collected. Total RNA was isolated using PureLink RNA Mini Kit (Thermo Fisher Scientific) according to the protocol of the manufacturer. Afterward, DNase I (Invitrogen; Waltham, MA, USA) was used to digest residual genomic DNA of samples, and then Turbo DNA-free Kit (Ambion; Austin, TX, USA) was used for DNA removal following the protocol of the manufacturer.

TABLE 1 | Primer list for RT-quantitative PCR (qPCR).

Gene	Primer	Sequence (5′ → 3′)	References
actβ	Forward	TGGCTGGGTGTTGAAGGTCT	Do Carmo et al. (2020)
	Reverse	AGCACGGCATCGTCACCAACT	
gapdh	Forward	CAACGACCACTTTGTCAAGC	Do Carmo et al. (2020)
	Reverse	TTCCTCTTGTGCTCTTGCTG	
muc2	Forward	CAGCACCGATTGCTGAGTTG	Do Carmo et al. (2020)
	Reverse	GCTGGTCATCTCAATGGCAG	
zo1	Forward	GAATGATGGTTGGTATGGTGCG	Do Carmo et al. (2020)
	Reverse	TCAGAAGTGTGTCTACTGTCCG	
zo2	Forward	GGAGACCAGATTCTGAAGGTGAACACA	Rabah et al. (2020)
	Reverse	CCTTTGGGGATTTCTAGCAGGTAGAGGAC	
cld-1	Forward	CTGGAAGATGATGAGGTGCAGAA	Rabah et al. (2020)
	Reverse	CTAATGTCGCCAGACCTGAA	
cld-5	Forward	ACGGGAGGAGCGCTTTAC	Pfeiffer et al. (2011)
	Reverse	GTTGGCGAACCAGCAGAG	
ocln	Forward	GGACCCTGACCACTATGAAACAGACTA	Rabah et al. (2020)
	Reverse	TAGGTGGATATTCCCTGACCCAGTC	
inos	Forward	CAGCTGGGCTGTACAAACCTT	Rabah et al. (2020)
	Reverse	CATTGGAAGTGAAGCGTTTCG	
pparg	Forward	CAGGCTTCCACTATGGAGTTC	PlÉ et al. (2016)
	Reverse	GGCAGTTAAGATCACACCTATCA	
il10	Forward	AAAGAAGGCATGCACAGCTC	Do Carmo et al. (2020)
	Reverse	AAGCATGTTAGGCAGGTTGC	
il17a	Forward	GCTCCAGAAGGCCCTCAGA	Rabah et al. (2020)
	Reverse	AGCTTTCCCTCCGCATTGA	

Note. muc 2, *Mucin 2;* zo1, zonula *Occludes 1;* zo2, zonula *Occludes 2;* cln-1, *Claudin-1;* cln-5, *Claudin-5;* ocln, *Occludin;* inos, *inducible nitric oxide synthase;* pparg, *peroxisome proliferator-activated receptor-gamma;* il10, *interleukin-10.,*

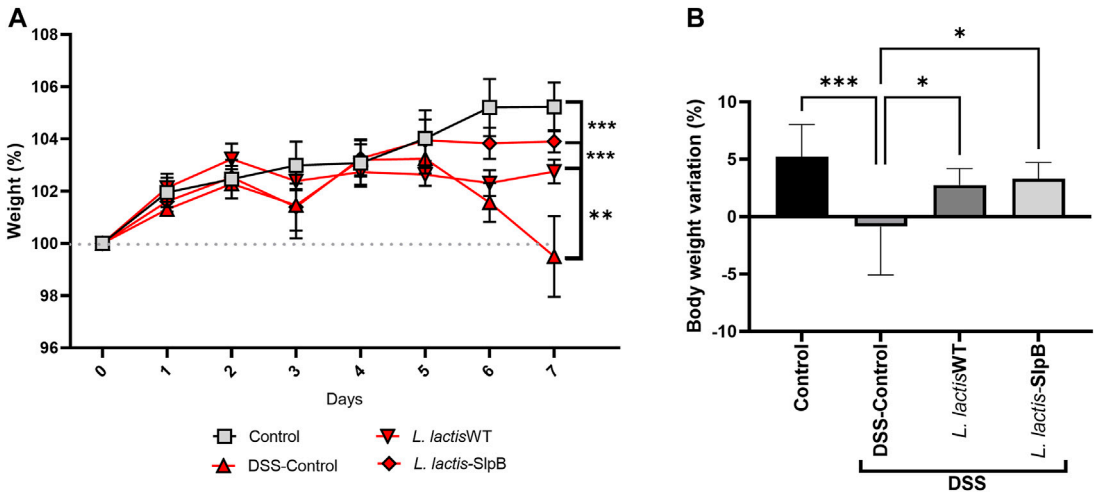

FIGURE 1 | *Lactococcus lactis*-Surface-layer protein (Slp)B is able to control weight loss in Dextran Sulfate Sodium (DSS)-induced colitis. Time-course of body weight during the seven experimental days **(A)** and weight loss **(B)** are shown. The two-way ANOVA **(A)**, one-way ANOVA **(B)**, and Tukey's *post-hoc* tests were used for the multiple comparisons (The data represent the mean ± SD of 12 mice per group). Asterisks represent statistically significant differences as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

RNA quality was checked by agarose gel and NanoDrop® ND-1000 (260/230 ratio). To obtain the samples cDNA the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems; Foster City, CA, United States) was used. Quantitative PCR (qPCR) was determined using iTaq universal SYBR green supermix (Biorad; Hercules, CA, United States) and gene specific primers (**Table 1**), for Mucin 2 (*muc-2*), Zonula occludens 1 (*zo-1*), zonula occludens 2 (*zo-2*), Claudin-1 (*cln-1*), Claudin-5 (*cln-5*), Occludin (*ocln*), inducible nitric oxide synthase (*inos*), peroxisome proliferator-activated receptor-gamma (pparg), and cytokine genes for interleukin-10 (*il-10*), *il-17*, as well as housekeeping genes encoding β-actin (*actβ*) and GAPDH (*gapdh*). The amplification cycles were performed as described: 95°C for 30 s, and 40 cycles of 95°C for 15 s and 60°C for 30 s on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Results were expressed as a fold-change of expression levels, using the mean and standard deviations of target expression ($2^{-\Delta\Delta Ct}$).

Cytokine Quantification by Enzyme-Linked Immunosorbent Assay

For the quantification of cytokines, the samples were weighed, and 50 mg of colon tissue was homogenized in 1 ml of PBS solution containing Tween-20 (0.05%) (Sigma-Aldrich, St. Louis, MO, USA), Phenylmethylsulfonyl fluoride (PMSF) 0.1 mM (Sigma-Aldrich, St. Louis, MO, USA), 0.1 mM benzethonium chloride (Sigma-Aldrich, St. Louis, MO, USA), 10 mM EDTA (Synth, São Paulo, São Paulo, Brazil), and aprotinin A 20 KIU (Sigma-Aldrich, St. Louis, MO, USA). The homogenized samples were then centrifuged at 3,000 × *g* for 10 min at 4°C, and the supernatants were collected to perform the enzyme-linked immunosorbent assay (ELISA). Plates were coated with purified monoclonal antibodies reactive with cytokines IL-1β, IL-10, IL-12, p70, IL-17, TGFβ1, and TNF-α (B&D Systems, Inc., USA), overnight at 4°C. Then, plate wells were washed, supernatants were added, and the plates were again incubated overnight at 4°C. On the third day, biotinylated monoclonal antibodies against cytokines (R&D Systems, Inc., USA) were added to the plates and incubated for 2 h at room temperature. Color was developed at room temperature with 100 µl/well of orthophenylenediamine (1 mg/m) and 0.04% (v/v) H_2O_2 substrate in sodium citrate buffer. The reaction was stopped by the addition of 20 µl/well of 2N H_2SO_4. The absorbance was measured at 492 nm using a Microplate Reader Model 680 (BIO-RAD).

Statistical Analyses

Data were analyzed using one-way ANOVA followed by Tukey's post-test and performed in GraphPad Prism version 9.1 for Windows (GraphPad Software, San Diego, CA, USA). The experimental assays were performed in triplicate, and the results were expressed as mean ± standard deviation. Asterisks demonstrated in all figures represent the significant differences between the experimental groups and were indicated as follows: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$.

RESULTS

Lactococcus lactis-Surface-Layer Protein B Reduces Weight Loss in Dextran Sulfate Sodium-Induced Colitis

Expression of the *P. freudenreichii* SlpB protein by *L. lactis* NCDO 2118 was first verified by Western blotting (**Supplementary Figure S2**). We then investigated the ability of

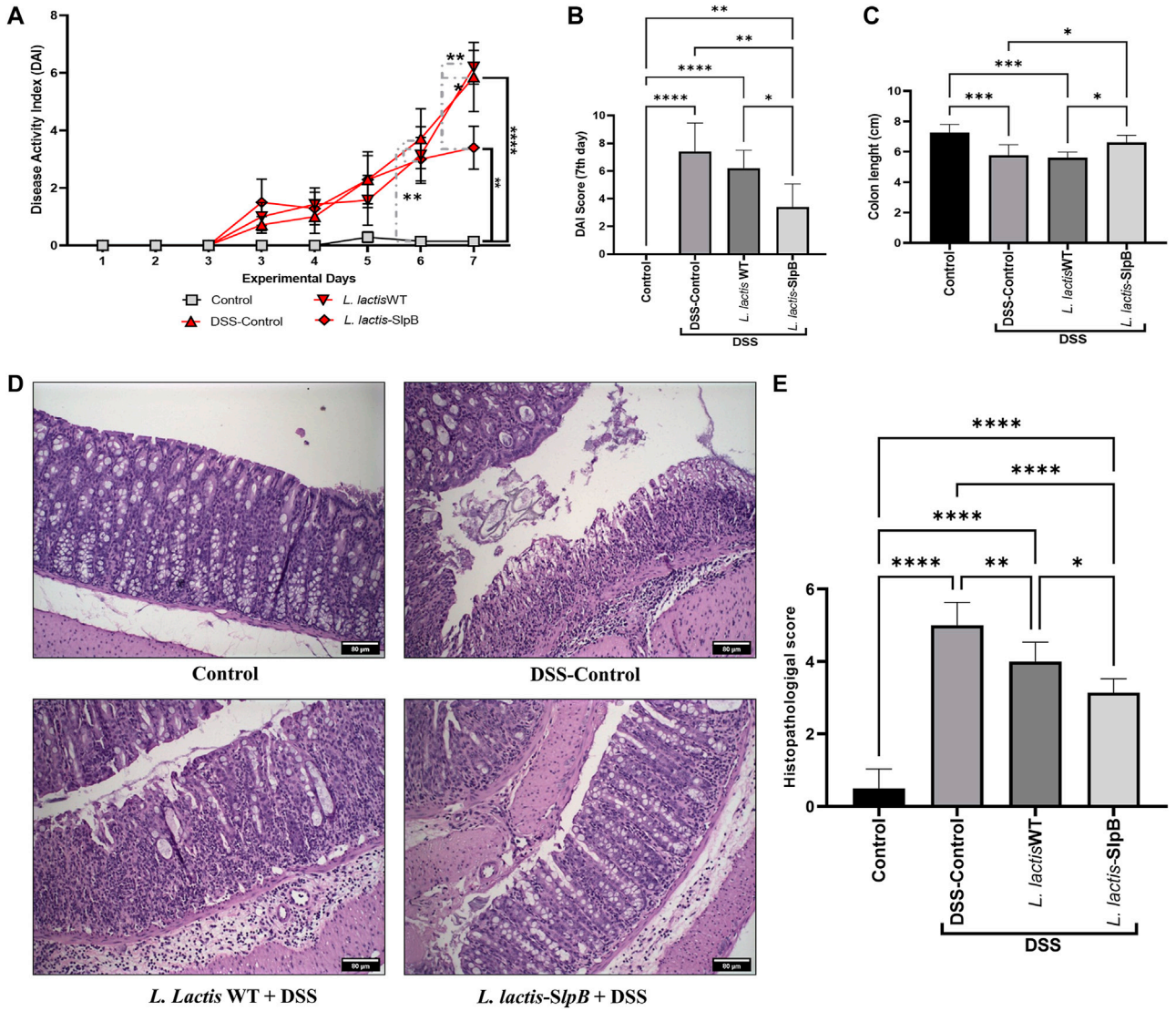

FIGURE 2 | *L lactis*-SlpB alleviates clinical symptoms in DSS-colitis mice model and reduces colon mucosal damage. Disease activity index over the seven experimental days **(A)**, at the last day **(B)**, Colon length analysis **(C)**, Micrograph images of the histopathological analysis of the colon tissue **(D)** and analysis of the histopathological score **(E)** are shown. The slides were stained in hematoxylin and eosin (H&E) and analyzed under ×20 magnification. The two-way ANOVA **(A)**, one-way ANOVA **(B)**, and Tukey's *post-hoc* tests were used for the multiple comparisons (The data represent the mean ± SD of 12 mice per group). Asterisks represent statistically significant differences as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

such expression to enhance the probiotic properties of the *L. lactis* in the context of DSS-colitis. First, the liquid and food consumption, as well as the weight of the animals, were monitored during the seven experimental days. In the DSS group, significant -weight loss was observed ($p < 0.001$ and $p < 0.0001$, respectively) on the sixth and seventh days (**Figures 1A, B**). However, at the end of the 7 days, both treatments with *L. lactis*-SlpB and the control group *L. lactis* WT were able to limit such weight loss of the animals ($p < 0.001$ and $p < 0.01$, respectively), when compared with the DSS group. Concerning liquid and food intake, no significant change was observed between experimental groups (**Supplementary Figure S3**).

Lactococcus lactis-Surface-Layer Protein B Alleviates Clinical and Macroscopic Symptoms in Dextran Sulfate Sodium-Colitis Mice Model

Regarding disease activity index (DAI) analysis (**Figures 2A, B**), as expected, the DSS significantly increased the score (5.85 ± 3.18) in the disease control group (DSS Control), when compared with the healthy group (Control) on the sixth and seventh days ($p < 0.05$ and $p < 0.0001$, respectively). At the end of 7 days, *L. lactis*-SlpB administration was shown to mitigate the signs of clinical colitis, based on DAI score (3.40 ± 1.67), when compared with the DSS control ($p < 0.01$). Treatment with *L. lactis* WT strain failed, by

contrast, to reduce DAI score in this DSS mice model (6.20 ± 1.30). Additionally, *L. lactis*-SlpB administration significantly limited the colon length shortening ($p < 0.05$) caused by the DSS administration, when compared with the DSS group (**Figure 2C**). It was more effective than *L. lactis* WT, which failed to do so (**Figure 2C**).

Surface-Layer Protein B Protein Improves the Potential of *Lactococcus lactis* to Reduce Colon Mucosal Damage

Histological analysis revealed that consumption of *L. lactis*-SlpB was able to mitigate the colon damage caused by DSS administration (**Figure 2D**). Precisely, it preserved colon morphological structure, reduced inflammatory cell infiltration in the lamina propria, submucosa, and muscular layer. Furthermore, animals that received *L. lactis* WT showed a slight decrease in mucosal damage, when compared with DSS control, but some ulcerations and a large infiltration of inflammatory cells were still observed. These results become evident through the analysis of the histopathological score, shown in **Figure 2E**, where, among the groups that consumed the DSS solution, the group *L. lactis*-SlpB showed significant differences in the histopathological score (3.14 ± 0.37), when compared with the group treated with *L. lactis* WT (4.00 ± 0.5, $p < 0.05$) and the DSS control group (5.0 ± 0.63, $p < 0.0001$). As expected, DSS-colitis induction resulted in a substantial decrease in goblet cells number in the DSS control group (67.08 ± 17.85 goblet cell/hpf). A significant increase in the number of goblet cells (**Figures 3A, B**) was exclusively observed in the group treated with *L. lactis*-SlpB (99.90 ± 20.51 goblet cell/hpf), when compared with the DSS control group ($p < 0, 05$). It was, however, not enough to re-establish the levels of the control group (133.1 ± 14.62 goblet cell/hpf, $p < 0.05$). In addition, *L. lactis* WT strain was not able to significantly increase the number of goblet cells (compared with the DSS-control group), and there was no statistical difference between *L. lactis*-SlpB and *L. lactis* WT strains (90.20 ± 6.64 goblet cell/hpf). A decrease in crypt depth (**Figure 3C**) was observed in the DSS Control group (191.1 μm ± 45.4, $p < 0.05$), compared with the Control group (232.3 μm ± 41.73). However, there was no statistical difference in the depth of the Crypts of Lieberkühn, between the treated groups *L. lactis* WT (212.5 μm ± 28.91) and *L. lactis*-SlpB (209.7 μm ± 46.30) with the control group (232.3 μm ± 41.73) and DSS group (191.1 μm ± 45.4).

Wild-Type and Recombinant Strains Both Reduce Levels of Myeloperoxidase Activity and Eosinophilic Peroxidase

Consumption of *L. lactis* WT and/or *L. lactis*-SlpB significantly decreased the amount of colon enzyme activity of MPO (57.27 ± 48.43 and 17.50 ± 6.65, respectively) (**Figure 4A**), with statistically significant differences for both treatments, when compared with the DSS control group (232.7 ± 115.9, $p < 0.0001$). The same scenario was repeated when the EPO enzyme activity was quantified (**Figure 4B**), where both strains, *L. lactis* WT and *L. lactis*-SlpB, proved effective to reduce EPO levels (0.07 ± 0.05 and 0.04 ± 0.04, respectively), with statistically significant differences, compared with the DSS Control group (0.28 ± 0.10, $p < 0.0001$). In addition, the results shown in **Supplementary Figure S4** demonstrate high levels of secretory IgA (sIgA) in the inflamed control group DSS (91.30 μg/ml ± 40.93). However, no statistical differences between the groups *L. lactis* WT (63.54 μg/ml ± 17.17) and *L. lactis-slpB* (59.44 μg/ml ± 26.85 and control group (62.81 μg/ml ± 28.17) was observed.

Lactococcus lactis-Surface-Layer Protein B Increases Expression of Genes Involved in Epithelial Barrier Protection

In the context of DSS-induced colitis, consumption of *L. lactis-slpB* increased significantly ($p < 0.001$) the colonic mRNA expression levels (**Figure 5**) of *muc-2* gene and epithelial barrier genes *zo-1, cln-1,* and *cln-5* (1.75 ± 0.87; 2.65 ± 0.72; 1.46 ± 0.54; 1.56 ± 0.35, respectively), when compared with the DSS Control group (0.60 ± 0.21; 0.97 ± 0.49; 0.41 ± 0.25; 0.90 ± 0.31, respectively). Interestingly, no difference in the expression levels of the *zo-2* gene was found between the experimental groups.

Pro and Anti-Inflammatory Genes Implicated in Ulcerative Colitis are Modulated by the *Lactococcus lactis*-Surface-Layer Protein B Recombinant Strain

The increase in the *inos* gene expression levels triggered by DSS administration in the inflammatory control group (8.31 ± 4.66) were controlled by the administration of *L. lactis*-SlpB strain (1.39 ± 1.08, $p < 0.01$) (**Figure 6A**). On the other hand, mRNA levels of *pparγ* were decreased in the DSS Control group (0.59 ± 0.20) and in the *L. lactis* WT group (0.58 ± 0.30), but the *L. lactis*-SlpB administration restored significant levels of *pparγ* colonic expression (1.07 ± 0.54, $p < 0.01$) (**Figure 6B**). Regarding the expression of genes encoding pro and anti-inflammatory cytokines (**Figures 6C, D**), *L. lactis*-SlpB group showed significantly reduced levels of *il-17* gene expression (0.39 ± 0.27, $p < 0.01$), compared with the DSS control group (1.83 ± 1.03). Finally, the gene expression of anti-inflammatory cytokine *il-10* was reduced in the DSS Control group (0.31 ± 0.11), but was restored in the group that received the *L. lactis*-SlpB strain (1.25 ± 0.55, $p < 0.001$). However, there were no statistical differences in the colonic expression levels of *il-17* and *il-10* cytokines, between the groups receiving *L. lactis* WT or *L. lactis*-SlpB.

Lactococcus lactis-Surface-Layer Protein B Strain Modulates Cytokine Production in the Mice Colon

Levels of colonic pro-inflammatory cytokines IL-17, IL-12, and TNF-α (13.66 ± 2.285, 20.17 ± 1.36; 17.41 ± 6.01, respectively) (**Figures 7A–C**) were increased in the DSS control group. In contrast, *L. lactis*-SlpB group showed significantly reduced levels of these cytokines, TNF-α, IL-17, and IL-12 (19.98 ± 7.04, $p < 0.05$;

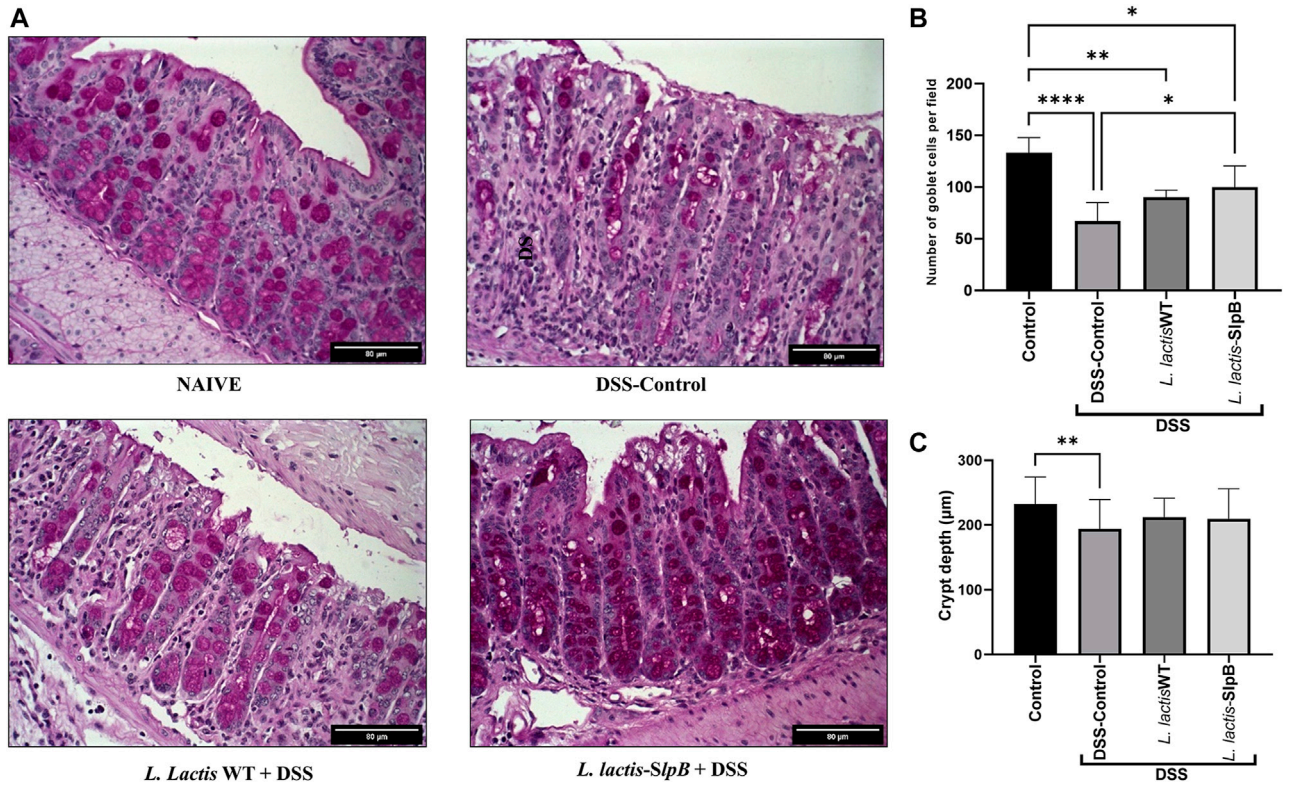

FIGURE 3 | *L. lactis*-SlpB mitigates histological signs of DSS-colitis. Model images of micrographs for analysis of goblet cells in colon tissue **(A)** and the result of goblet cell quantification by field **(B)** and depth of colon intestinal crypts **(C)** are shown. The slides were stained in Periodic Acid-Schiff (PAS), goblet cells have intense purple-pink tones, and analyzed under ×40 magnification. The data represent the mean ± SD of 12 mice per group. One-way ANOVA and Tukey's *post-hoc* tests were used for multiple comparisons. Asterisks represent statistically significant differences as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

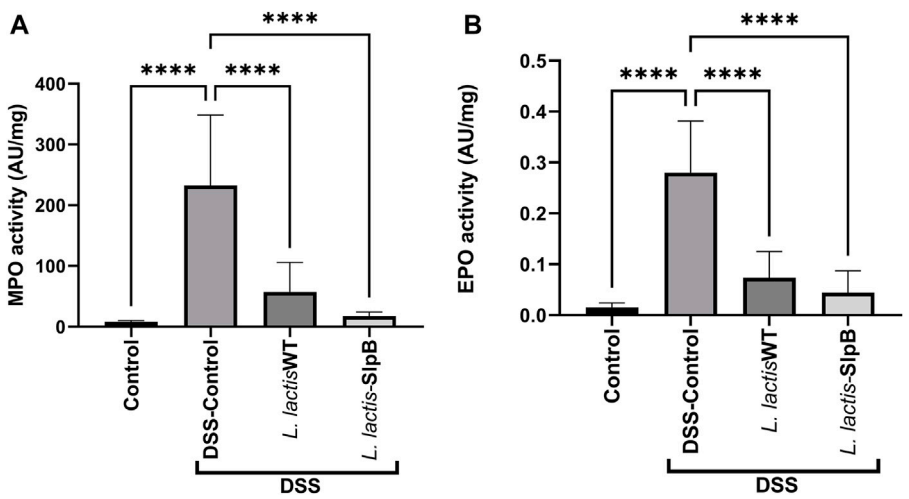

FIGURE 4 | *L. lactis* Wild-type and *L. lactis*-SlpB strains prevent DSS-induced increase of myeloperoxidase (MPO) and eosinophilic peroxidase (EPO) activity. Quantification of the myeloperoxidase [MPO, **(A)**] and eosinophilic [EPO, **(B)**] enzymes in the colon tissue is shown. One-way ANOVA and Tukey's *post-hoc* tests were used for multiple comparisons. The data represent the mean ± SD of six mice per group. Asterisks represent statistically significant differences as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

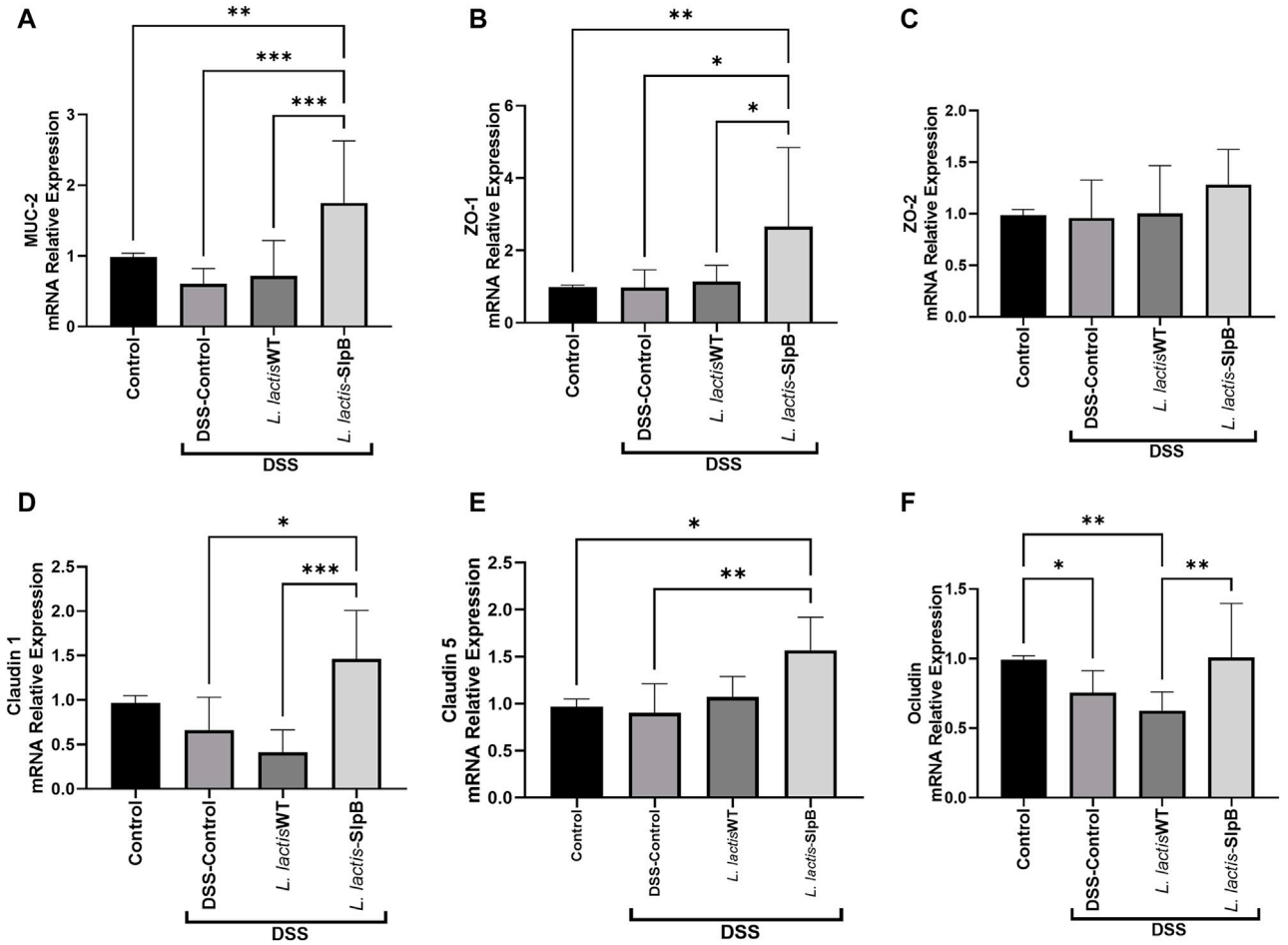

FIGURE 5 | *L. lactis*-SlpB increases the expression of genes involved in epithelial barrier protection. Quantification of the expression of the genes Mucin 2 (*muc2*) **(A)**, Zonula Occludes 1 (*zo-1*) **(B)**, Zonula Occludes 2 (*zo-2*) **(C)**, Claudin-1 (*cln-1*) **(D)**, Claudin-5 (*cln-5*) **(E)**, and Occludin (*ocln*) **(F)**, in the mice colon, is shown. One-way ANOVA and Tukey's *post-hoc* tests were used for multiple comparisons. The data represent the mean ± SD of six mice per group. Asterisks represent statistically significant differences as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

10.51 ± 2.20, $p < 0.05$; 14.20 ± 1.20, $p < 0.05$, respectively), compared with the DSS control group. In addition, an increase in the colonic levels of IL-10 and TGF-β cytokines (**Figures 7D, E**) was observed in the group treated with *L. lactis*-SlpB (59.10 ± 23.14; 468.70 ± 261.60, $p < 0.05$, respectively) compared with the DSS Control group (31.96 ± 4.91; 183.80 ± 63.11, respectively). However, *L. lactis*-SlpB failed to decrease levels of Il-1β induced by DSS (**Figure 7F**).

DISCUSSION

Ulcerative colitis (UC) is an inflammatory bowel disease, which can be mimicked using *in vivo* models through induction with chemicals such as DSS (Wirtz et al., 2017). The inflammation occurring in UC affects the colonic epithelial cells and results in an impairment in the mucosal barrier function. In addition, colitis is marked by obvious clinical signs, such as weight loss, diarrhea, and occult blood in the feces (Zhang and Li, 2014). Some strains of lactic acid bacteria, such as *L. lactis* NCDO2118 (Luerce et al., 2014) and propionibacteria, such as *P. freudenreichii* CIRM-BIA 129 (Rabah et al., 2020) have already given promising results in alleviating the symptoms of UC. Precisely, *P. freudenreichii* 129 expresses a surface protein SlpB protein, which can be directly linked to its probiotic effects (Rabah et al., 2020). Thus, the DSS-induced mice model, the molecular tools for *L. lactis* NCDO 2118 to produce recombinant protein, and the SlpB protein from the *Pf* 129 strain, constitute the perfect scenario to test the potential of this protein to enhance the probiotic effects of other strains.

Several proteins expression models have already been successfully developed for *L. lactis* strains (Tavares et al., 2020). The xylose-induced model (XIES), developed exclusively for *L. lactis* NCDO 2118 by Miyoshi et al. (2004), not only expresses but also uses the mechanism of secretion and targeting of the protein to the extracellular medium, allowing the correct targeting of the SlpB surface protein. Besides, due to simple metabolism and rapid growth (12–24 h), in contrast to *P. freuderinchii* 2–3 days to reach the stationary growth phase, *L. lactis* began to be used for the production of recombinant

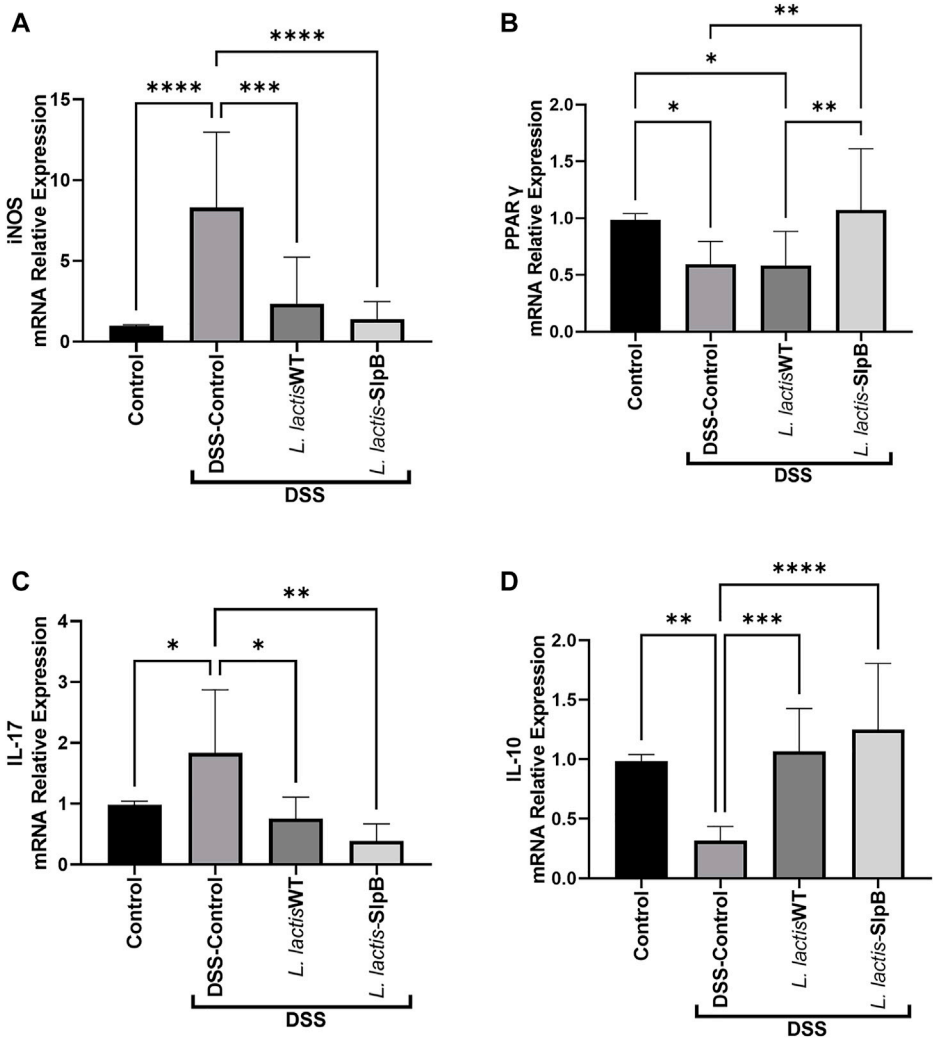

FIGURE 6 | *L. lactis*-SlpB strain modulates expression of pro and anti-inflammatory genes implicated in ulcerative colitis. Quantification of the expression of the genes inducible nitric oxide synthase (*inos*) **(A)**, peroxisome proliferator-activated receptor-gamma (*pparg*) **(B)**, as well as interleukin-17 (*il-17*) **(C)** and interleukin-10 (*il-10*) **(D)** pro and anti-inflammatory cytokines, is shown. The data represent the mean ± SD of six mice per group. One-way ANOVA and Tukey's *post-hoc* tests were used for multiple comparisons ($n = 6$). Asterisks represent statistically significant differences as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

proteins in the cytoplasm or secreted into the extracellular medium (Carvalho et al., 2017).

L. lactis NCDO 2118 and *P. freudenreichii* CIRM-BIA 129, as well as the action of some surface proteins in a purified way, can bring relief in weight loss in mice with colitis or other models of inflammation (Luerce et al., 2014; Cai et al., 2018; Do Carmo et al., 2020; Rabah et al., 2020). Luerce et al. (2014) showed that the *L. lactis* NCDO2118 strain can prevent colon shortening in the context of colitis. In our work, mitigation of inflammation was enhanced by the presence of SlpB. Indeed, concerning colon length, the wild-type strain alone did not show efficacy in this DSS mice model. Furthermore, although *L. lactis* NCDO2118 WT gave good results by decreasing the histopathological score, in accordance with Luerce et al. (2014), our results indicate that the presence of the SlpB protein further enhanced the histopathological score, when compared with the *L. lactis* NCDO 2118 group. The preservation of goblet cells is an important aspect of probiotic mechanisms of action. These cells produce mucus, which serves as a barrier preventing the direct adhesion of microorganisms to the epithelium (Abrantes et al., 2020). It is worth noting that the *L. lactis*-SlpB strain increased the expression of the *muc*-2 gene and restored goblet cells in animals treated with DSS. The goblet cells are responsible for producing the mucus that covers the intestinal mucosa, and high levels of sIgA can be found in the mucus layer of the intestine in healthy individuals (Rogier et al., 2014). However, increased IgA secretion may be related to an inflammatory response caused by disturbances in the ileum intestinal barrier, as shown by Rabah et al. (2020). In the present work, only the DSS group exhibited high levels of sIgA, but no significant differences between groups were found. Moreover, preservation of the epithelium, demonstrated in the histology of animals that received *L. lactis*-SlpB, is consistent with the increased

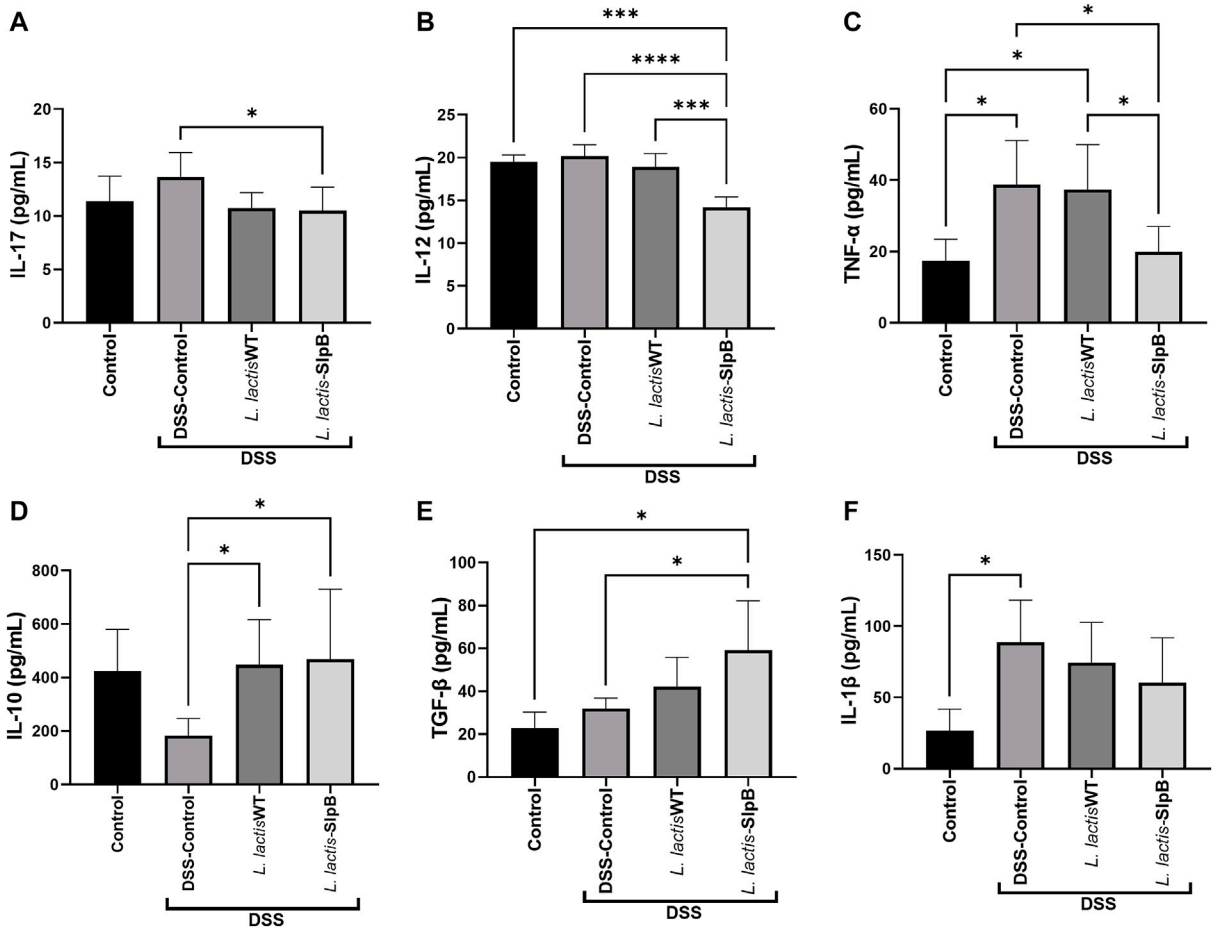

FIGURE 7 | *L. lactis*-SlpB strain modulates cytokines production in the mice colon. Colonic cytokines concentrations levels of IL-17 **(A)**, IL-12 **(B)**, TNF-α, **(C)**, IL-10 **(D)**, TGF-β **(E)**, IL-1β **(F)** were quantified by enzyme-linked immunosorbent assay (ELISA). The data represent the mean ± SD of six mice per group. One-way ANOVA and Tukey's *post-hoc* tests were used for multiple comparisons. Asterisks represent statistically significant differences as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

expression of the *zo-1*, *cld-1*, *cld-5*, and *ocln* genes responsible for the expression of tight junction proteins, maintaining the epithelial barrier function and controlling cell permeability (Landy et al., 2016). It is plausible that the SlpB protein plays a central role in reinforcing the epithelial barrier, but further studies are needed, such as treatment with purified SlpB protein and monitoring of intestinal permeability, to conclude this statement.

Regarding the inflammatory cells infiltrate, it was visibly attenuated in the group treated with *L. lactis* NCDO2118 and even smaller in the animals treated with *L. lactis*-SlpB. This infiltrate is composed of mononuclear and polymorphonuclear cells, and in ulcerative colitis, increased levels of neutrophils and eosinophils are mainly observed (Villanacci et al., 2013). Therefore, we quantified the colonic activity of myeloperoxidase (MPO) and eosinophilic peroxidase (EPO), as a means of indirect determination of neutrophils and eosinophils, respectively, in the colon of animals. The results obtained in the quantification of MPO and EPO enzymes in this work corroborate those described by Han et al. (2021), where the DSS group enhanced activity of both enzymes in the colon.

PPARγ is a regulator of intestinal inflammation. It inhibits transcription of pro-inflammatory cytokine genes, such as *ifn-γ*, and the inducible nitric oxide synthase (*inos*) gene (Dubuquoy et al., 2006; Rabah et al., 2020). The activation of the *inos* expression is responsible for mediating the accumulation of nitric oxide that results in oxidative stress and it is directly linked to gastrointestinal immunopathology, such as ulcerative colitis (Kolios et al., 2004). We observed that the DSS-induced colitis resulted in a significant increase in the expression of nitric oxide synthase corroborating the results obtained in the work of Rabah et al. (2020). However, *L. lactis*-SlpB triggered an increase in the expression of the *pparγ* gene, showing an effect not found with the administration of the *L. lactis* NCDO 2118 wild-type strain. Patients with ulcerative colitis have impaired expression of *pparγ* in the colon and the increased expression of this gene can lead to the inhibition of inflammatory cytokines such as IL-1β and TNF-α (Dubuquoy et al., 2006). We accordingly observed a

reduction in the levels of TNFα in the animals treated with the *L. lactis*-SlpB, where the *L. lactis* NCDO 2118 wild-type strain failed to prevent the increase in cytokine secretion caused by DSS-induced colitis.

Immune response, i.e., pro and anti-inflammatory cytokines, is one of the main mediators of the pathogenesis of colitis (Ko and Auyeung, 2014). In this aspect, bacterial surface proteins may moderate dysregulation of cytokines, as demonstrated by the effects of the SlpA protein from *Lactobacillus acidophilus* CICC in the DSS-induced colitis model (Cai et al., 2018). The impact of the SlpB protein on the IL-12 cytokine expression has already been demonstrated by Do Carmo et al. (2020). Indeed, consumption of the wild strain of *Pf* 129 triggered a decrease in this cytokine during 5-FU-induced mucositis, but the same was not observed as a result of the consumption of the knockout strain for the *slpB* gene (*Pf* 129Δ*slpB*). Furthermore, activation of IL-10 and TGF-β secretion by the *L. lactis*-SlpB strain was also observed here, an important factor that contributes to the attenuation of the inflammatory response in the colon caused by DSS. The anti-inflammatory cytokine IL-10 can inhibit the production of IL-1β, IL-6, and TNF-α. However, to access IL-10 protective effect against colitis, IL-10 signaling pathway must be triggered before the induction of DSS colitis (Li and He, 2004). The mechanism of action of the SlpB protein also appears to be aimed at limiting the inflammatory process, mainly containing IL-17, *via* regulation of Th17 cells. Colliou et al. (2017) demonstrated that propionibacteria that enrich the microbiota of infants through breastfeeding can attenuate the incidence of necrotizing enterocolitis through the regulation of Th17 cells.

CONCLUSION

S-layer proteins have a great potential to mediate host–probiotic interactions *via* intestinal cells, which are important to maintain gut immunity homeostasis and to mitigate inflammatory diseases. Mice that received *L. lactis*-slpB showed a significant reduction in colitis severity symptoms. Thus, it is plausible that the presence of SlpB protein in the *L. lactis* NCDO 2118 strain increased its potential to control the effects and symptoms of DSS-induced colitis, such as decreased DAI. Further studies involving purified SlpB protein and its expression in other organisms are needed to unravel its ability to enhance probiotic effects. This work demonstrates that *L. lactis* NCDO 2118 harboring SlpB recombinant protein prevents the inflammatory process during DSS-induced colitis in mice, opening perspectives for the development of new probiotic functional foods for personalized nutrition in the context of IBD.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee on Animal Experimentation of the Universidade Federal de Minas Gerais (CEUA-UFMG, Brazil) 148/2020.

AUTHOR CONTRIBUTIONS

GB, VA, and FC conceived and designed the experiments. BFC, MB, and AG-G were major contributors to animal experimentation. BGC and FM contributed with the necessary help to carry out the MPO, EPO, and sIgA quantification assays. EO and SS contributed mainly to the performance of the *in vitro* assays. EF performed, analyzed, and interpreted the histological analysis from colon slides. GB, VA, and FC wrote the original draft. GJ and YL gave scientific advice and participated in the writing of the manuscript. All authors contributed to the data interpretation, drafting of the manuscript, critically revising the manuscript, and approving its final 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/fphar.2021.755825/full#supplementary-material

SUPPLEMENTARY FIGURE S1 | Experimental design to explore anti-inflammatory effects of L. lactis-SlpB in DSS-colitis mice model. C57BL6 mice were divided into 4 groups and receiving or not different treatments for 7 days simultaneous colitis induction by 1.7% DSS in drinking water for 7 days prior to euthanasia. Different disease parameters were monitored to study the severity of colitis.

SUPPLEMENTARY FIGURE S2 | Western Blotting detection of surface layer protein SlpB. PVDF membranes were treated using rabbit antibodies raised against P. freudenreichii 129 surface layer protein SlpB. **A-** Positive control - surface proteins (including SpB) from the Pf 129 strain extracted by Guanidine Chloride (20 ug of proteins). L. lactis-SlpB non-induced (Growth with Glucose-plasmid repressor): **B-** Total protein (10 µg protein); **C-** Supernatant protein (10 ug of proteins), D- Surface proteins (Guanidine Chloride Extract - 50 ug of proteins L. lactis-SlpB induced by Xylose: **E-** Total protein (10 µg protein); **F-** Supernatant protein (10 ug of proteins), **G-** Surface proteins (Guanidine Chloride Extract - 50 ug of proteins).

SUPPLEMENTARY FIGURE S3 | (A) Liquid intake (ml/mice) and (B) Food consumption (g/mice) during experimental procedure of DSS colitis induction. Results were expressed as means ± standard deviations (SD). No significant differences were found.

SUPPLEMENTARY FIGURE S4 | L. lactis Wild-type and L. lactis-SlpB strains did not alter the secretory IgA production. The concentration of secretory IgA in the small intestine. The data represent the mean ± SD of 12 mice per group. The One-Way ANOVA and Tukey post-hoc tests were used for the multiple comparisons. No significant difference was found.

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