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Elucidating the Connection Between the Health-Promoting Properties of *Limosilactobacillus fermentum* Lf2 and Its Exopolysaccharides

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Abstract: The potential probiotic properties of *Limosilactobacillus fermentum* Lf2, an exopolysaccharide (EPS)-producing strain, were assessed in C57BL/6 mice. The aim of this work was to elucidate if these properties could be associated with the ability to produce EPSs. Mice were divided into three treatments: L (mice treated with Lf2), E (animals that received EPSs), and C (control group). The levels of fecal acetic and propionic acids significantly increased in L and E compared with C. Catalase activity increased in L in comparison with the other groups in the liver and small intestine. The enzyme activities of superoxide dismutase and glutathione S-transferase increased in the large intestine for L compared with C. In addition, in the large intestine, the concentration of TNF-α was reduced in L and E in comparison with C. In the small intestine, TNF-α, IFN-γ, IL-12, and IL-6 presented lower levels in L and E than C. The analysis of the gut microbiota showed that L presented higher levels of *Peptococcaceae* and *Rikenellaceae*, while E had higher levels of *Peptococcaceae* than C. Overall, these results provide new insights into the relationship between the probiotic properties of lactic acid bacteria and their ability to produce EPSs.

Keywords: lactic acid bacteria; probiotic properties; exopolysaccharides; oxidative stress; immunomodulation; gut microbiota; short-chain fatty acids

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1. Introduction

Fermented foods have been shown to have enhanced nutritional and health-promoting properties due to the transformation of different substrates and concomitant formation of bioactive or bioavailable end-products [1]. Bioactive compounds can derive from protein, lipid, and carbohydrate catabolism during the fermentation process; for instance, the production of vitamins and antioxidants has been reported for several lactic acid bacteria (LAB), in particular, members of the *Lactobacillaceae* family [2]. Bioactivities related to ameliorating metabolic syndromes, lowering blood pressure and cholesterol, anti-cancer effects, and immune response enhancement have been widely described [3].

Probiotics are microorganisms usually used or found in food fermentation processes and are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [4]. Some of them are able to produce exopolysaccharides (EPSs), microbial polymers that have been widely studied in the field of pharmacological applications due to their antiallergic, anticoagulant, antithrombotic, immunomodulatory, and blood cholesterol-lowering properties, among others [5]. The linkage between the probiotic properties of certain LAB and their ability to produce these molecules has not been entirely elucidated, but there are some recent studies addressing this question. In this direction, Sungur et al. [6] studied the antitumor effects of EPS-producing strains of *Lactobacillus gasseri* isolated from the human vagina and found that both live bacteria and their EPSs were able to inhibit the cell proliferation of cervical cancer cells (HeLa), with the impact of the freeze-dried EPSs being strain-dependent. The authors concluded that the diversity in the sugar composition of EPSs could contribute to their adhesion and proliferation properties. Moreover, Taj et al. [7] evaluated the probiotic potential of novel EPS-producing strains of *Streptococcus thermophilus* isolated from Dahi sold in different local markets of Pakistan. In vitro studies revealed that these strains presented resistance to harsh conditions (bile salt resistance and acid tolerance tests), good cell surface hydrophobicity, auto-aggregation, and co-aggregation (especially against *Listeria monocytogenes*).

Limosilactobacillus fermentum Lf2 (Lf2) is an autochthonous strain isolated from Argentinian cheese, and it has been demonstrated to produce large amounts of EPS (~2 g L^{-1}) [8]. The purified EPS extract is composed of a high-molecular-mass β-glucan (1.23×10^6 g mol^{-1}) and two medium-molecular-mass polysaccharides (with an average weight mass of 8.8×10^4 g mol^{-1}). The β-glucan presented immunomodulatory properties in peripheral blood mononuclear cells since it was able to modulate proinflammatory cytokines, such as TNF-α [9]. This ingredient also had protective effects against *Salmonella* infection and showed enhanced intestinal IgA secretion in mice [8]. Additionally, the EPS evidenced a symbiotic effect when combined with *Bifidobacterium animalis* subsp. *lactis* INL1 (a probiotic strain isolated from breast milk) [8]. More recently, the connection between the health-promoting properties of Lf2 and its EPS was elucidated in a mouse model of chronic colitis [10]. The effects observed for the strain were similar to those of the purified EPS in terms of immune response, antioxidant properties, and microbiota modulation, suggesting that the potentially probiotic properties of Lf2 could be associated with its capacity to produce these metabolites.

Although Lf2 and its EPS have shown positive health effects in a chronic colitis mouse model, there is a lack of information about their impact on non-challenged animals. In this context, the aim of this work was to assess the probiotic properties of Lf2 and to elucidate the relationship with its ability to produce these molecules using naïve mice. The antioxidant properties in the liver and intestines, the production of fecal short-chain fatty acids (SCFAs), the immunomodulatory effects, and the impact on the gut microbiota composition were determined for both the strain and its purified EPS. All these parameters are critical indicators of the overall impact on health, especially on the organs that are directly affected by the administration of functional food ingredients. The analyses carried out in this study covered, from different angles, properties of great importance when addressing the characterization of new potential probiotics and prebiotics.

2. Materials and Methods

2.1. Organisms and Growth Conditions

Limosilactobacillus fermentum Lf2 (INLAIN collection) was kept at −80 °C in MRS broth (Biokar, Beauvais, France) with 15% (*v*/*v*) glycerol. The strain was routinely grown in MRS broth (37 °C; 18 h; aerobic conditions).

2.2. EPS Production

EPS was obtained and purified according to Ale et al. [10]. Briefly, fermentations were carried out in a 2 L fermenter (Sartorius Biostat A plus®, Goettingen, Germany) at 30 °C and pH 6.5 for 48 h with agitation and CO_2 sparging. The bacteria were then removed by centrifugation, and the EPSs were recovered by precipitation with chilled ethanol (Cicarelli, Santa Fe, Argentina). The precipitate was collected by centrifugation, dissolved in double-distilled water, and dialyzed using 12–14 kDa MWCO membranes (Sigma Aldrich, St. Louis, MO, USA) against distilled water (4 °C; 72 h). The frozen EPS solution was freeze-dried, and further purification was performed with DNAse I (5 mg mL^{-1}, Sigma Aldrich, St. Louis, MO, USA) and Pronase E (50 mg mL^{-1}, Roche, Mannheim, Germany). A precipitation step with TCA (12%, *w*/*v*) and neutralization with NaOH were carried out, and the suspension was dialyzed against distilled water. This solution was kept at −80 °C until freeze drying to obtain the purified EPS extract.

2.3. Mouse Model

Eight-week-old female C57BL/6 mice of 19–21 g were obtained from the animal facility of the Facultad de Ciencias Exactas y Naturales (FCEN, UBA (Universidad de Buenos Aires), Argentina). The animals were treated according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH 8023, 1978), and the procedures were approved by the Committee of Ethics, Safety, and Hygiene in Experimental Work (CEySTE, CONICET, Argentina). The mice received sterile tap water and a balanced diet (Cooperación, Buenos Aires, Argentina) ad libitum. After 1 week of adaptation, the animals were administered with the following treatments by gavage for 15 days (10 mice/group): (i) control mice treated with 10% *w/v* lactose (group C); (ii) mice that received Lf2 in 10% lactose (1×10^8 CFU/mouse/day) (group L); and (iii) mice treated with the purified EPS extract from Lf2 in 10% lactose (1.2 mg/mouse/day, equivalent to 60 mg/kg/day) (group E). According to preliminary studies that showed the good viability of this strain when freeze-dried in 10% lactose, this matrix was selected as the carrier for all the treatments so they could be comparable.

2.4. Determination of s-IgA and Cytokines

Once the treatments finished, the animals received an anesthetic cocktail (0.3 mL/mouse), which consisted of 1.8 mL of ketamine (50 mg mL^{-1}, KetonalTM, Richmond Vet Pharna, Buenos Aires, Argentina), 0.9 mL of 2% xylazine (Alfasan, Santa Fe, Argentina), and 0.3 mL of acepromazine (10 mg mL^{-1}, Acedan, Hollyday Scott, Buenos Aires, Argentina) to a final volume of 10 mL, adding 7 mL of sterile saline solution. The cocktail was kept at 4 °C until use. The mice were sacrificed by cervical dislocation, and s-IgA and cytokine levels were determined as previously described in [10].

The small intestine was recovered and flushed with 5 mL of cold PBS buffer containing 1% of a protease inhibitor cocktail (Sigma-Aldrich). This fluid was centrifuged (10,000× *g*; 10 min; 4 °C), and the supernatant was stored at −80 °C for s-IgA quantification. Portions of the distal small intestine (jejunum and ileum) and the whole large intestine were removed for homogenate preparations. The tissues were kept frozen (−80 °C) immediately after the extractions and then resuspended (100 mg of tissue/mL) in an extraction buffer (100 mL of PBS, 0·293 g of EDTA, and 50 µL of Tween 20) containing 1% of a protease inhibitor cocktail, homogenized (Ultra-turrax T8, IKA Labortechnik, Staufen, Germany), and centrifuged (10,000× *g*; 10 min; 4 °C).

The concentration of s-IgA and the levels of cytokines IL-10, IL-12, TNF-α, IFN-γ, IL-6, and IL-2 were analyzed with the corresponding mouse ELISA sets (BD OptEIA, BD, Biosciences PharMingen, San Diego, CA, USA).

2.5. Oxidative Stress in Liver and Intestines

The livers and intestines were kept frozen at −80 °C until analysis. The extracts for measuring the activities of antioxidant enzymes were prepared by homogenization with phosphate buffer (pH 6.5), according to Bacchetta et al. [11]. The activity of superoxide dismutase (SOD) was determined by its ability to inhibit epinephrine autoxidation [12]. Catalase (CAT) activity was measured following the decomposition of H_2O_2 [13], and glutathione S-transferase activity (GST) was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate [14]. From the same tissues, another homogenization was prepared with 20% trichloroacetic acid (TCA) and 4% butylhydroxytoluene (BHT) to measure the lipid peroxidation levels (LPO) in the liver and small and large intestines through the formation of thiobarbituric acid-reactive substances (TBARSs) [15]. All determinations were performed in triplicate and expressed in terms of the sample protein [16].

2.6. Determination of Organic Acids in Feces

The determination of acetic acid, propionic acid, butyric acid, and lactic acid in feces was carried out before and after each treatment using high-performance liquid chromatography (HPLC) [10]. Briefly, the samples were resuspended in 0.01 M H_2SO_4 (1:10) (mobile phase) and centrifuged for 10 min at 10,000× *g*. The supernatant reached pH 2 with a fixed volume of 2 M H_2SO_4, and it was treated at 65 °C for 20 min. After centrifugation (16,000× *g*; 30 min), the supernatant was filtered (0.45 µm membranes, Millipore, São Paulo, Brazil) and injected into the chromatographic system.

The HPLC system (Perkin Elmer, Norwalk, CT, USA) consisted of a quaternary pump, an online degasser, a column oven, and two in-line detectors: UV–visible (set at 210 nm) and refractive index (set at 35 °C). The column oven and the RI were Flexar Series, while the rest of the components were 200 Series. The organic acids were identified in both detectors but only quantified using the RI detector. The elution was performed isocratically at 65 °C using 0.01 M H_2SO_4 at a flow rate of 0.6 mL min^{-1} and an Aminex HPX-87H column (300 × 7.8 mm) equipped with a cation H+ microguard cartridge (Bio-Rad Laboratories, Hercules, CA, USA). Analytical-grade reagents (Sigma Aldrich, USA) were used as standards to obtain the calibration curves.

2.7. Analysis of Gut Microbiota

DNA extraction was carried out with the QIAamp Fast Stool DNA Mini Kit (Qiagen, Hilden, Germany), and the 16S rRNA gene (regions V3–V4) was amplified. The amplicons were then sequenced with the Illumina MiSEQ platform (ABIMO-IB, INTA-CONICET, Buenos Aires, Argentina) and analyzed using the QIIME2 pipeline (v2022.8, [17]). The reads were checked with dada2 [18], and the amplicon sequence variants (ASVs) were used for alpha and beta diversity analyses. The taxonomic classification was obtained by comparing all the ASVs against the SILVA database (release 138, [19]).

2.8. Statistical Analysis

A one-way ANOVA was used to compare the treatments, and the Kruskal–Wallis test was applied when the assumptions for the ANOVA were not satisfied (Infostat, version 2020). When the differences were significant ($p < 0.05$), Tukey and Dunn post hoc tests were used, respectively. Three replicates of analysis were considered for all the determinations. Principal component analysis (PCA) was performed using the fviz_pca_biplot function from the factoextra R package (https://www.r-project.org/), accessed on 15 March 2024.

3. Results

3.1. Determination of s-IgA and Cytokines

The immunomodulatory effects of both the EPS-producing strain Lf2 and its purified EPS were assessed in the large and small intestines. In the large intestine, the concentrations of IFN-γ, IL-2, IL-10, and IL-12 showed no significant differences between the treatments (Figure 1a), while the levels of the pro-inflammatory cytokine TNF-α were significantly decreased in E and L to the same extent. In the small intestine, the treatments E and L presented concentrations of IFN-γ, IL-6, and TNF-α that were significantly lower than in the control group, and the levels of IL-2 remained similar (Figure 1b). Additionally, as shown in Figure 1c, the concentrations of IL-12 and IL-10 were reduced and increased ($p < 0.05$), respectively, in L compared with C. Although the same tendency was observed for E, the differences were not significant. Regarding the levels of IgA in the intestinal fluid, all the groups presented similar values. From these results, a relationship between the strain and its EPS could be suggested in terms of their immunomodulatory effects.

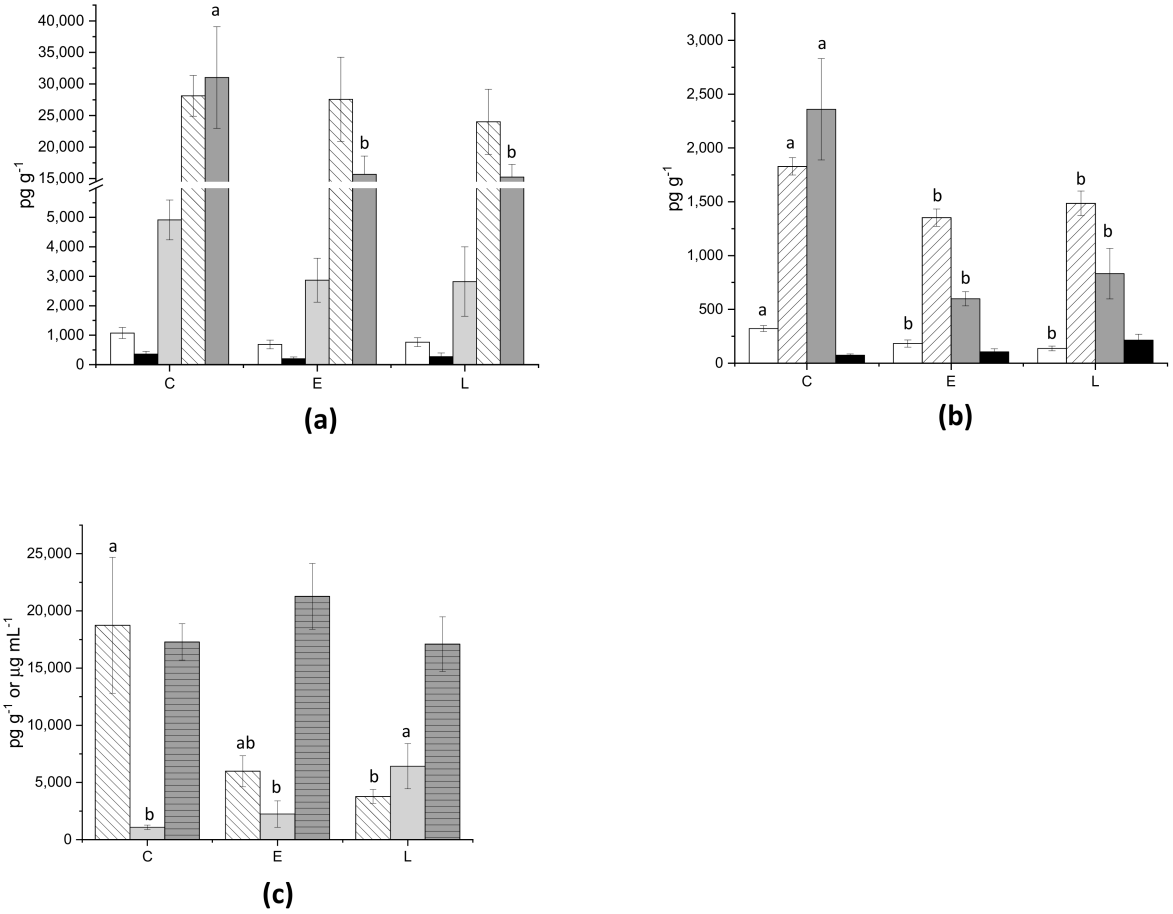

Figure 1. Concentrations of cytokines in the large and small intestines. (**a**) Levels of cytokines in the large intestine: IFN-γ (□), IL-2 (■), IL-10 (▭), IL-12 (▨), and TNF-α (▭); (**b**) concentrations of cytokines in the small intestine: IFN-γ (□), IL-6 (▨), TNF-α (▭), and IL-2 (■); (**c**) concentrations of cytokines (pg g^{-1}) and IgA (µg mL^{-1}) in the small intestine: IL-12 (▨), IL-10 (▭), and IgA (▭). C: control group; E: mice that received purified EPS from Lf2; L: mice that received Lf2. Results are expressed as $\bar{x} \pm$ SEM. Different letters represent significant differences ($p < 0.05$) between treatments, and only significant differences are indicated.

3.2. Oxidative Stress in Liver and Intestines

The CAT activity in the liver and small intestine was significantly increased in L in comparison with C, while in the large intestine, the enzyme activity was similar among

the treatments (Figure 2a). In the large intestine, the enzyme activity of SOD presented the highest values in L ($p < 0.05$), and the activity of GST was significantly higher in L compared with C (Figure 2b,c). Meanwhile, GST and SOD activities remained similar among treatments in the liver and small intestine (Figure 2b,c). No significant differences were observed in lipid peroxidation (Figure 2d). Overall, it seems that the antioxidant properties might be related to the strain, with no evident correlation with the purified EPS extract.

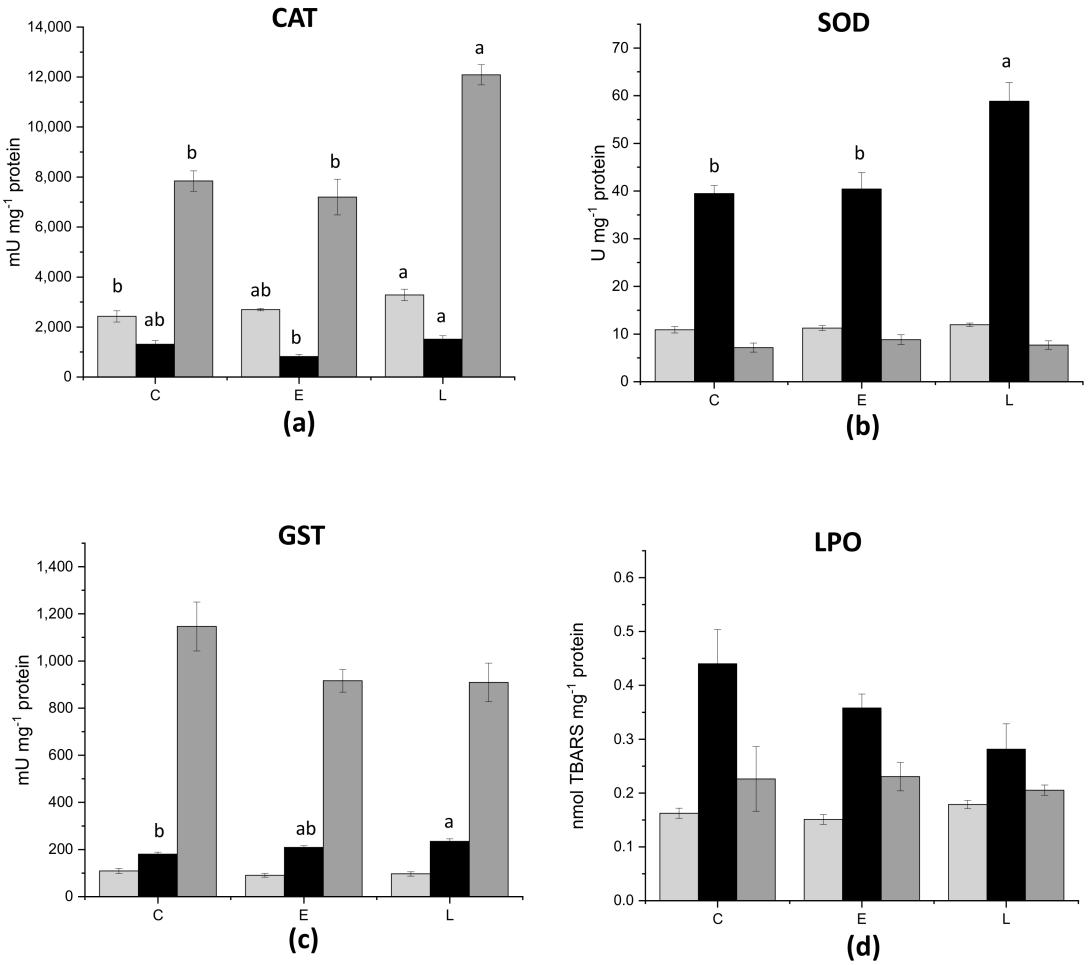

Figure 2. Enzyme activity and lipid peroxidation levels in liver (▭), large intestine (■), and small intestine (▭). (**a**) CAT; (**b**) SOD; (**c**) GST; (**d**) LPO. C: control group; E: mice that received purified EPS from Lf2; L: mice that received Lf2. Results are expressed as $\bar{x} \pm$ SEM. Different letters represent significant differences ($p < 0.05$) between treatments, and only significant differences are indicated.

3.3. Determination of Organic Acids in Feces

The levels of SCFAs and lactic acid are shown in Figure 3, from which the concentration at the beginning of each treatment was subtracted for each mouse. Significant differences were observed for the levels of acetic acid and propionic acid, as the concentrations obtained for both L and E were higher than for C ($p < 0.05$). Meanwhile, the levels of butyric acid were similar among treatments, while the concentration of lactic acid was significantly higher in E compared with C. Similar to the results obtained for the immune response, the profiles of the organic acids were similar between the strain and its EPS, suggesting that these metabolites might provide the strain with the ability to modulate the gut microbiota.

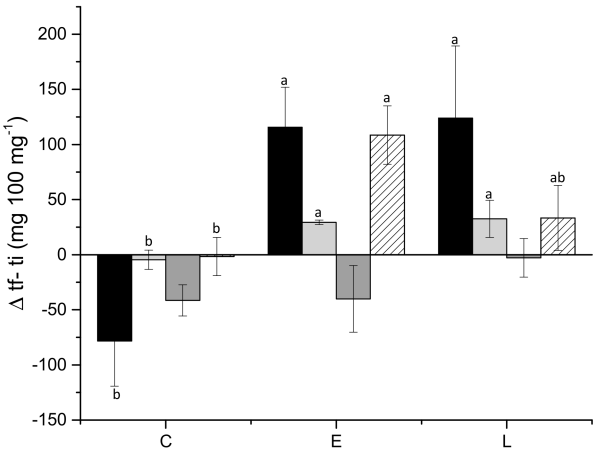

Figure 3. Concentrations of organic acids in feces (the levels at t0 were subtracted). Acetic acid (■), propionic acid (▭), butyric acid (▬), and lactic acid (▨). C: control group; E: mice that received purified EPS from Lf2; L: mice that received Lf2. Results are expressed as $\overline{x} \pm$ SEM. Different letters represent significant differences ($p < 0.05$) between treatments, and only significant differences are indicated.

3.4. Analysis of Gut Microbiota

The barplot in Figure 4 shows the relative abundances of the bacterial groups at the end of the treatments. It can be observed that no differences were visually evident between the groups, with *Bacteroidales* being the most abundant order, followed by *Lachnospirales*. Nevertheless, significant differences ($p < 0.05$) were appreciated for three taxonomic groups at 15 days, which are shown in Table 1. Both E and L presented an increased abundance of *Peptococcaceae* compared with the control group, while L presented the highest levels of *Rikenellaceae* ($p < 0.05$). Additionally, the abundance of *Saccharimonadaceae* between C and each treatment was statistically similar, with the differences being significant only between E and L ($p < 0.05$). Meanwhile, both the α- and β-diversity indexes (Shannon and Bray–Curtis indexes, respectively) showed similar values ($p > 0.1$) in terms of treatment and time.

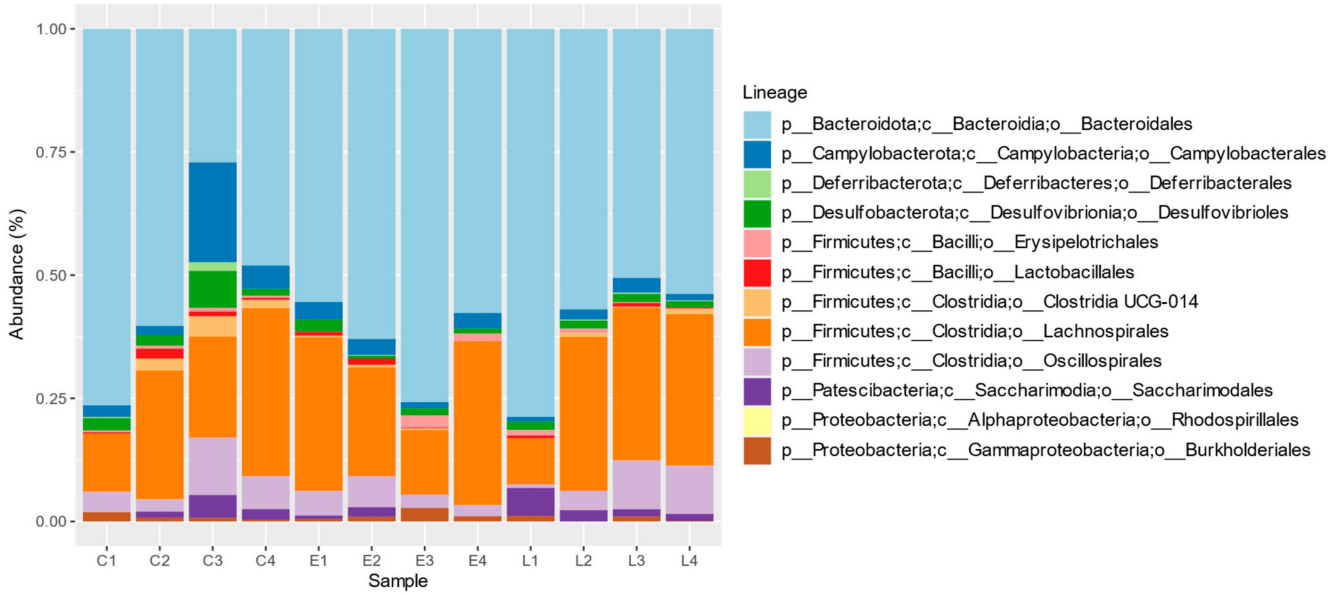

Figure 4. Barplot representing the relative abundances of the bacterial orders and lineages at the end of the treatments.

Table 1. Median values of the absolute abundances of bacterial families that showed significant differences ($p < 0.05$) at the end of the treatments.

Taxa	C	E	L	*p*-Value
Bacteria_Firmicutes_Clostridia_Peptococcales_Peptococcaceae	0 [b]	10 [a]	19.5 [a]	0.0155
Bacteria_Bacteroidota_Bacteroidia_Bacteroidales_Rikenellaceae	114.5 [b]	94 [b]	193.5 [a]	0.0231
Bacteria_Patescibacteria_Saccharimonadia_Saccharimonadales_Saccharimonadaceae	90 [a,b]	16 [b]	134 [a]	0.0349

C: control group; E: mice that received purified EPS from Lf2; L: mice that received Lf2. Different letters represent significant differences ($p < 0.05$) between treatments.

3.5. Multivariate Analysis

A multivariate analysis was performed to evaluate the distribution of the treatments according to the parameters studied (immune response, oxidative stress parameters, and organic acids profile) (Figure 5). When the absolute abundance of all the bacterial taxonomic groups was included, the clustering remained similar. The PCA biplot shown in Figure 5 (PC2 vs. PC1) explained 49.9% of the overall variance. It can be clearly observed that the control group was separated from the L and E treatments along PC1, while the L and E groups were separately distributed by PC2. The control group was related to most proinflammatory cytokines in both the large and small intestines. Mice that received the strain were positively correlated with the activity of antioxidant enzymes in the liver and small and large intestines, as expected. This group was also associated with IL-10 levels in the small intestine and butyric acid. Meanwhile, the E treatment was related to SCFAs, especially acetic and propionic acids, in accordance with the results observed in Section 3.3.

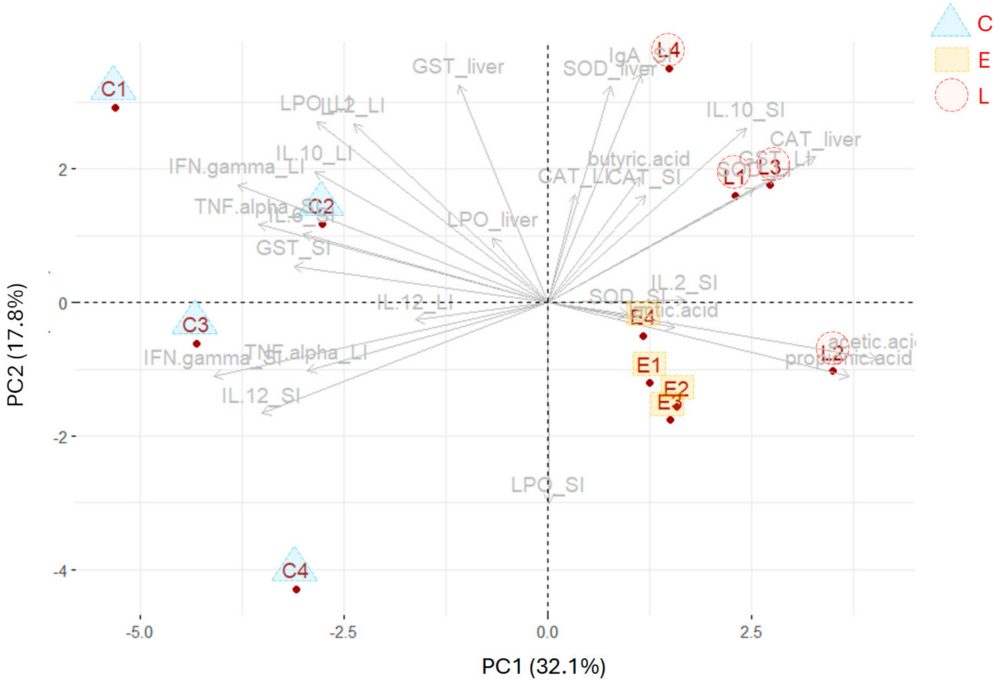

Figure 5. Principal component analysis including the variables related to the immune profiles, oxidative stress, and SCFAs. SI: small intestine; LI: large intestine.

4. Discussion

In this work, the impact of Lf2 and its EPS was assessed in vivo, with the aim of elucidating a possible link between them using a naïve mouse model. The EPS produced by this strain is of interest because the metabolites have demonstrated several technological and health-promoting properties [8].

Regarding the immune response, the results obtained showed that most of the positive effects took place in the small intestine for both the L and E treatments, as the levels of IFN-γ, IL-6, TNF-α, and IL-12 were significantly decreased compared with the control group. Additionally, in this tissue, the regulatory cytokine IL-10 presented a higher concentration in L in comparison with the control group. Meanwhile, the levels of IgA from the intestinal fluid had no significant differences between treatments, while in the large intestine, only TNF-α presented significant differences, with its concentration being the highest in C ($p < 0.05$).

In a previous study by our group, a similar approach was taken but using a TNBS (trinitrobenzene sulfonic acid)-induced chronic colitis mouse model [10]. In this case, a significant increase in IgA levels was observed for the group that received EPS from Lf2 and a decrease in IFN-γ levels in the small intestine for the group treated with Lf2. In the large intestine, IL-2 and IFN-γ presented the lowest levels in the groups treated with EPS and the strain. Although the anti-inflammatory effects were not as evident as the ones obtained in the present work, similar to the results presented, the concentrations of proinflammatory cytokines were decreased in the groups treated with EPS and Lf2, suggesting, again, a potential relationship between the health-promoting properties of the strain and the EPS produced, especially for non-challenged mice.

In the current literature, there are several studies on potential probiotic strains of the species *Limosilactobacillus fermentum* or their EPSs, but, as far as we are aware, only a few studies addressing the properties of both the strain and its purified EPS are available. In this direction, Kim et al. [20] characterized the strain *Limosilactobacillus fermentum* KGC1601 isolated from *Panax ginseng* and its EPS. Through qRT-PCR and ELISA assays, the authors confirmed that EPS purified from the culture media, as well as the culture media of *L. fermentum* KGC1601, had anti-inflammatory effects on RAW264.7 cells.

In terms of the antioxidant properties, in general, it was observed that the treatment with Lf2 was the most effective in enhancing the activity of antioxidant enzymes (CAT, SOD, and GST), especially in the large intestine. Also, CAT activity was increased in the liver and small intestine in this group. Meanwhile, the activities of the antioxidant enzymes in the C and E groups remained similar ($p > 0.05$). In this direction, Bhawal et al. [21] investigated the physicochemical characteristics and antioxidative role of a cell-bound exopolysaccharide (EPS-b) from *Limosilactobacillus fermentum* MTCC 5898. The authors found that the pretreatment of Caco-2 cells with this molecule maintained the basal activity of antioxidant enzymes (SOD, CAT, and glutathione peroxidase) similar to the control in the presence or absence of H_2O_2-induced stress. These results are in line with the ones found in this study for those mice that received the purified EPS fraction since this group was statistically similar to the control treatment. It should be noted that EPS-b is composed of glucose and galactose subunits, like the EPS from Lf2 [8], suggesting a potential relationship between chemical structure and functionality. Future studies addressing the impact of structure and composition on health-promoting properties are necessary to confirm this hypothesis.

In another work, Ayyanna et al. [22] studied the probiotic strains *Limosilactobacillus mucosae* AN1 and *Limosilactobacillus fermentum* SNR1 in carrageenan (acute) and complete Freund's adjuvant-induced inflammation (chronic) models. These strains were administered orally to Wistar male rats as microencapsulated and whole cells. The results showed that the groups that received the probiotics (both encapsulated and unencapsulated) exhibited low levels of lipid peroxidation and higher antioxidant enzyme activities (GST, CAT, and SOD) in comparison with the control and inflammation control tissues. These results are in line with the findings of the present work for Lf2.

The determination of organic acids in feces also showed significant differences between treatments. Their quantification is a relevant indicator of the gut microbiota ecosystem since they are the main final metabolic products of carbohydrate fermentation [23]. Acetic and propionic acids presented higher levels in E and L compared with the control group ($p < 0.05$) at the end of the treatment, while E had the highest levels of lactic acid. The concentration of butyric acid remained similar among groups. Even though lactic acid is not considered a SCFA, it is one of the most relevant fermentation products of LAB. The fermentation products of some LAB species can participate as intermediate metabolites in the metabolic pathways of other bacterial species. In this direction, it is known that lactate, pyruvate, and ethanol are utilized for SCFA synthesis [24].

The species *Limosilactobacillus fermentum* has been associated with an increase in fecal SCFAs. For instance, de Luna Freire et al. [25] assessed the effects of a mixed formulation containing three different strains of this species on the metabolic and immune parameters of female Wistar rats fed a high-fat diet. The authors found that the administration of these strains increased acetate and succinate fecal contents and reduced hyperlipidemia and hyperglycemia in rats subject to this diet. Regarding the effects observed for the EPS extract, a previous study by our group showed that the administration of yogurt containing EPSs from Lf2 increased the levels of fecal acetic and butyric acids in a mouse model (male BALB/c mice), results that were associated with a higher abundance of SCFA-producing bacteria in the gut [8]. These findings are in line with the ones obtained for the present work, even though a different matrix was used.

Increased concentrations of SCFAs have been described for EPSs from other LAB genera, such as *Lacticaseibacillus rhamnosus*. Zhu et al. [26] identified four EPSs with different molecular compositions from *Lacticaseibacillus rhamnosus* ZFM231. The authors found that these purified EPS fractions could be fermented to produce SCFAs by the gut microbiota in human fecal samples from twelve healthy volunteers. It seems that these molecules can be utilized by the gut microbiota, contributing to its balance and leading to an increase in health-promoting metabolites. Nevertheless, it should be noted that this property needs to be evaluated for each strain and under determined growth conditions, as several factors could impact the composition (and, consequently, the functional properties) of EPSs.

In terms of microbiota composition, the effects of both the strain and EPS were mild, probably because a naïve mouse model was used. Recently, when a TNBS-induced colitis mouse model was used for the same treatments, more significant changes were observed at the end of the assay [10]. In the present study, the family *Peptococcaceae* significantly increased in both E and L compared with the control group (Table 1). Although the information about this bacterial family is scarce, some positive properties have been described. For example, Lan et al. [27] investigated the association between the gut microbiota composition and leukemia and evaluated the potential protective effect of the gut microbiota on developing this condition. The multivariable Mendelian randomization (MVMR) study showed a protective effect of the *Clostridia* class, *Peptococcaceae* family, *Clostridiales* order, and *Firmicutes* phylum. In another study in which a similar approach was applied, the family *Peptococcaceae* was also associated with a protective role in the development of benign gastric tumors [28]

In addition, *Rikenellaceae* presented the highest abundance in L ($p < 0.05$). It was reported that members of this family might play a role as adiposity modulators through the production of acetate and propionate [29], SCFAs that were significantly increased in mice fed the strain. Although these organic acids also presented higher values than the control group in mice fed the EPS ($p < 0.05$), no significant differences were observed for

this bacterial family between E and C, probably because other bacteria were responsible for this increase in E.

In a recent study, the gut microbiota profile was characterized in a cohort of 201 Italian elderly subjects [30]. The authors reported that the highly diverse structures of the gut microbiome of the elderly could contribute to a reduced amount of visceral adipose tissue. In particular, the families *Christensenellaceae*, *Porphyromonadaceae*, and *Rikenellaceae* could play a protective role in cardiovascular and metabolic diseases associated with visceral fat and, thus, high amounts of these bacterial groups could be potential markers of healthy aging and longevity. Additionally, another study reported that the abundance of *Rikenellaceae* was significantly higher in lean than obese subjects [31].

Finally, the abundance of *Saccharimonadaceae* in both E and L groups remained similar to the control treatment. Nevertheless, L presented significantly higher values than E. This family was positively correlated with immune response parameters in cyclophosphamide-treated mice [32]. Meanwhile, the high abundance of this group was associated with the gut microbiota composition of mice with induced lupus [33], so more information is required to evaluate the overall effect that modifications in the abundance of this family could have.

In a nutshell, considering the results obtained, and as clearly shown by the PCA, a correlation between the properties of Lf2 and its purified EPS fraction could be suggested. This finding could be useful when addressing treatments for immunocompromised patients since probiotic strains could be causative agents of adverse effects, such as opportunistic infections [34]. Thus, by utilizing the purified EPS with similar health-promoting properties, these risks could be avoided.

Furthermore, the application of EPSs as food ingredients is more versatile than using the producing strain, as they might not negatively impact the final characteristics of the product, especially because extremely low concentrations can provide both technological properties and health benefits [35]. In addition, the preparation of EPS-rich postbiotics using EPS-producing LAB could be an attractive research area [36]. Finally, the direct application of Lf2 would be more economical and less time-consuming, and, at the same time, this strain could also prevent oxidative stress, according to the results obtained in this work. So, the choice of whether to use Lf2, its purified EPS, or an EPS-rich postbiotic extract (by inactivating the strain) will depend on each particular situation, and clinical trials will be necessary to confirm the health effects.

5. Conclusions

From the results obtained, it can be appreciated that the EPS might play a crucial role in the health-promoting properties of Lf2 in terms of the regulation of pro-inflammatory cytokines in the small and large intestines and the modulation of the gut microbiota (including the production of SCFAs). This correlation was not evident for the antioxidant enzymes' activities since their increase was only observed in the group that received the strain in all the tissues assessed. In order to confirm the relationship between the beneficial properties of Lf2 and its EPS, future studies comparing the effects between the wild-type strain and a mutant incapable of producing EPS could be undertaken. These findings could be useful when deciding whether to use this strain or its EPS depending on the application and could provide insight into potential criteria for the evaluation of new probiotic strains.

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Informed Consent Statement: Not applicable.

Data Availability Statement: All the raw data used in this study were deposited in the NCBI database as part of the bioproject PRJNA1063264 (SRR31596367 to SRR31596378). The data that support the findings are available from the corresponding author upon reasonable request.

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