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Protective Role of *Limosilactobacillus fermentum* Lf2 and Its Exopolysaccharides (EPS) in a TNBS-Induced Chronic Colitis Mouse Model

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Abstract: *Limosilactobacillus fermentum* Lf2 (Lf2) is an autochthonous strain that produces high levels of exopolysaccharides (EPS). The objective of this work was to evaluate the probiotic potential of Lf2 and its relationship with these metabolites in a mouse model of TNBS (trinitrobenzene sulfonic acid)-induced chronic colitis. Mice were treated intrarectally with increasing doses of TNBS resuspended in 50% ethanol for 14 days. In parallel, they received different treatments by gavage (lactose 10% as the matrix): freeze-dried Lf2 (L); purified EPS (E); and lactose 10% (T). A healthy control group (H) was treated with 50% alcohol without TNBS (intrarectally) and 10% lactose (by gavage). In the small intestine, there was a significant increase in IgA levels for the group that received EPS and a decrease in IFN-γ for mice treated with the strain compared to the other groups. In the large intestine, IL-2 and IFN-γ presented the lowest levels in the groups treated with EPS and the strain. The concentrations of acetic and propionic acids in mice that received Lf2 were the highest, while the levels of butyric acid were comparable to the healthy control group. An increase in the abundance of SCFA-producing bacteria was observed for mice treated with EPS and the strain in comparison with the colitis control group. The enzyme activity of catalase was higher in all the treatments compared to the TNBS-induced colitis control mice. To summarize the results obtained, a principal component analysis (PCA) was performed, clearly grouping the treatments in different clusters according to the variables studied. This is one of the first studies to address the role of a potential probiotic strain in a chronic colitis mouse model, trying to elucidate the relationship between its properties and the EPS synthesized.

Keywords: exopolysaccharides; lactobacilli; chronic colitis; intestinal microbiota; antioxidant enzymes; immunomodulation

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1. Introduction

Inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease, comprises conditions characterized by chronic and remittent gastrointestinal inflammation. IBD has a high prevalence in Western societies and newly industrialized nations [1]. The pathogenesis of IBD is complex and multifactorial; genetic, environmental, and microbial factors can lead to an imbalance of the mucosal immune response, negatively affecting the gut microbiota [2]. At present, conventional treatments aim at ameliorating symptoms with pharmacotherapy (aminosalicylates, corticosteroids, immunomodulators, and biologics) and other measures if necessary. However, as many patients do not respond to available treatments, new strategies are needed [3]. It is widely known that a healthy microbiota is

closely associated with normal gut functions and immunity, while dysbiosis is related to the onset and maintenance of IBD [4]. Therefore, targeting this dysbiosis is key to managing this condition.

Lactic acid bacteria (LAB) have played a crucial role in the manufacture of fermented food, and numerous strains have been associated with health-promoting properties [5,6]. According to Hill et al. [7], probiotics can be defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host". The mechanisms by which probiotics exert health benefits are not always clear, but their metabolites seem to be involved in some cases. In this direction, the majority of commercialized probiotic microorganisms for consumption by humans and animals are LAB, some of them being EPS (exopolysaccharides)-producers, suggesting that some of their health-promoting properties could be due to the release of these carbohydrates [8]. Synthesis of exopolysaccharides by LAB contributes to improving the sensory and rheological properties of the final products. Furthermore, EPS can be considered postbiotic ingredients if their benefits are demonstrated [9]. Among their health benefits, modulation of the immune system, anti-oxidative, anti-inflammatory, anti-microbial, anti-tumoral, and cholesterol-lowering activities, together with modulation of the gut microbiota, could be mentioned [10].

Many clinical and preclinical studies aim to demonstrate the impact of probiotics on IBD. Recently, a meta-analysis was carried out to explore the clinical effects and regulation of the gut microbiota when using probiotics, prebiotics, and synbiotics in IBD [11]. While prebiotics are substrates selectively utilized by host microorganisms, conferring a health benefit [12], synbiotics are a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confer a health benefit on the host [13]. The authors found that the treatment of IBD (especially ulcerative colitis) with synbiotics was more effective, and probiotics from the genera *Lactobacillus* and *Bifidobacterium*, or with more than one strain, were beneficial for IBD remission. Amongst the prebiotics informed, inulin, fructooligosaccharides (FOS), lactulose, and oligofructose-enriched inulin were included. As far as we are concerned, there is no clinical study addressing the effects of EPS from LAB on colitis-related conditions, with the most beneficial properties demonstrated only in preclinical assays [14,15]. Nevertheless, the linkage between these molecules and the producing bacteria has not been elucidated yet.

Limosilactobacillus fermentum Lf2 (Lf2) is a strain isolated from Argentinian cheese, and it is able to synthesize large amounts of EPS (~2 g L^{-1}) under optimized conditions [16]. It was found that the purified EPS extract was mainly composed of a high molecular mass β-glucan (1.23×10^6 g mol^{-1}) and two medium molecular mass polysaccharides (average weight mass of 8.8×10^4 g mol^{-1}). The composition of the EPS depended on the culture conditions, since under statistically optimized growth conditions, the medium molecular mass molecules represented more than 75% of the mixture [17], while the high molecular mass β-glucan predominated under non-optimized conditions. Additionally, the β-glucan demonstrated immunomodulatory properties in peripheral blood mononuclear cells, as it modulated proinflammatory mediators (such as TNF-α), suggesting that it may reduce the risk of digestive diseases such as Crohn's disease and ulcerative colitis [18]. The EPS crude extract from Lf2 also had protective effects against *Salmonella* and promoted IgA secretion in the intestines of mice [19]. These EPS presented a synbiotic effect when co-administered with *Bifidobacterium animalis* subsp. *lactis* INL1, a probiotic autochthonous strain [20].

Regarding the technological properties, the EPS extract provided low-fat yoghurts with enhanced firmness and consistency, reduced syneresis, and acceptable organoleptic properties [21]. In a recent study, yoghurts made with Lf2 together with the starter culture increased the release of bioactive peptides in yoghurt and had a significant impact on the levels of organic acids and carbohydrates, rheological properties, and microstructure [22]. It should be noted that these effects were not observed when the purified EPS extract was used alone.

Given that interesting results were obtained when Lf2 was used in yogurt and considering that it would be more economical to use the strain to synthesize EPS in situ rather than

producing this purified ingredient, the present work was focused on demonstrating the functional properties of Lf2 in a colitis-induced mouse model, aiming at an understanding of the relationship between its ability to produce EPS and its health-promoting effects. In this context, the immunomodulatory activity, gut microbiota modulation, short-chain fatty acid production in cecum, and antioxidant properties were analyzed in a TNBS-induced chronic colitis mouse model.

2. Materials and Methods

2.1. Organisms and Growth Conditions

Limosilactobacillus fermentum Lf2 (INLAIN collection) was kept at −80 °C in MRS (De Man, Rogosa, and Sharpe, Biokar, Beauvais, France) broth with 15% (*v*/*v*) glycerol (Cicarelli, Santa Fe, Argentina). The strain was routinely grown in MRS broth (37 °C, 18 h, aerobic conditions).

2.2. Production of Exopolysaccharides

Exopolysaccharides from Lf2 were obtained under optimized conditions [16]. Briefly, fermentations were carried out in a 2 L fermenter (Sartorius Biostat A plus®, Goettingen, Germany) at 30 °C, pH 6.5, for 48 h with agitation and CO_2 sparging. Then, bacteria were removed by centrifugation (19,000× *g*, 30 min, 5 °C), and EPS were recovered by precipitation with two volumes of chilled ethanol (Cicarelli, Santa Fe, Argentina) at 4 °C for 48 h. The precipitate was collected by centrifugation (4000× *g*, 30 min, 5 °C), dissolved in double distilled water, and dialyzed against distilled water (4 °C, 72 h), using 12–14 kDa MWCO membranes (Sigma Aldrich, St. Louis, MO, USA). The frozen EPS solution was freeze-dried in crystallization dishes for 24 h at 0.0010 mBar and −56 °C (Alpha 1–4 LD Plus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), and further purification was performed [23]. Briefly, the purification steps comprised treatments with DNAse I (5 mg mL^{-1}, Sigma Aldrich) and Pronase E (50 mg mL^{-1}, Roche, Mannheim, Germany). Then, a precipitation step with TCA (12%, *w*/*v*) and neutralization with NaOH were performed, 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. Chronic Colitis Model

Six-week-old male BALB/c mice of 19–21 g were obtained from the Instituto de Ciencias Veterinarias del Litoral (ICiVet-Litoral, UNL-CONICET, Esperanza, Santa Fe, Argentina). The animals were treated following the National Institutes of Health guide for the care and use of Laboratory animals (NIH 8023, 1978), and all the procedures were previously approved by the Committee of Ethics, Safety, and Hygiene in Experimental Work (CEySTE, CONICET, Santa Fe, Argentina). Mice received sterile water and a balanced diet (Cooperación, Buenos Aires, Argentina) ad libitum. The chronic colitis model consisted of the administration of 3 increasing doses of TNBS with an interval of one week (n = 8 mice/group), according to Burns et al. [24]. Briefly, anesthetized mice received intrarectally 50 µL of TNBS (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.9% NaCl/ethanol (50/50 *v*/*v*) at a dose of 25 mg kg^{-1} (day 0), 37.5 mg kg^{-1} (day 7), and 80 mg kg^{-1} (day 14). In parallel, mice were administered with the following treatments: (i) Control healthy mice (50% ethanol instead of TNBS) + intra-gastric administration of lactose 10% *w*/*v* (group H); (ii) Control TNBS-treated mice + intra-gastric administration of lactose 10% (group T); (iii) TNBS-treated mice + intra-gastric administration of Lf2 in lactose 10% (3×10^8 CFU/mouse/day) (group L); and (iv) TNBS-treated mice + intra-gastric administration of purified EPS extract from Lf2 in lactose 10% (0.6 mg/mouse/day, equivalent to 30 mg/kg/day) (group E). Lactose was used as the carrier for all the oral treatments since the strain was freeze-dried in this matrix. This way, the effects observed in each group could be properly compared.

2.4. Microbiota Analysis

DNA extraction was performed with the QIAamp Fast Stool DNA Mini kit (Qiagen, Hilden, Germany) to amplify the 16S rRNA gene (regions V3–V4). Amplicons were sequenced using the Illumina MiSEQ platform (ABIMO-IB, INTA-CONICET, Buenos Aires, Argentina), generating 250 bp paired end reads. These sequences were analyzed using the QIIME2 pipeline [25]. Briefly, reads were quality checked using dada2 [26], and the resulting ASVs (Amplicon Sequence Variants) were used for alpha and beta diversity analysis. Lastly, the taxonomic classification was obtained by comparing all ASVs against the SILVA database [27].

2.5. Determination of Translocation, s-IgA, and Cytokines

After the treatments, animals received an anesthetic cocktail (0.3 mL/mouse). This solution was prepared according to Ale et al. [20] with 1.8 mL of ketamine (50 mg mL^{-1}, Ketonal^{TM}, Richmond Vet Pharna, Argentina), 0.9 mL of xylazine 2% (Alfasan, Argentina), and 0.3 mL of acepromazine (10 mg mL^{-1}, Acedan, Hollyday, Argentina) to a final volume of 10 mL, adding 7 mL of sterile saline solution; it was kept at 4 °C until the moment of application. Mice were sacrificed by cervical dislocation. Part of the liver was removed and homogenized in sterile PBS and plated onto violet red bile agar (VRBL, Biokar, Pantin, France) (37 °C, 24 h, aerobiosis) to evaluate translocation. s-IgA and cytokine levels were determined as previously described [19]. s-IgA concentration was determined in intestinal fluid, and cytokines IL-12, TNF-α, IFN-γ, IL-6, and IL-2 were determined in the distal small intestine (jejunum and ileum) with mouse ELISA Sets (BD OptEIA, BD, Biosciences PharMingen, San Diego, CA, USA). To obtain the intestinal fluid, the small intestine was flushed with 5 mL of cold PBS buffer containing 10 g L^{-1} protease inhibitor cocktail (Sigma-Aldrich). Then, this fluid was centrifuged (10,000× *g*, 10 min, 4 °C), and the supernatant was stored at −80 °C.

2.6. Oxidative Stress

Livers were kept frozen at −80 °C until analysis. Extracts for measuring the activities of antioxidant enzymes were prepared by homogenization with a phosphate buffer (pH 6.5) by a 10,000× *g*-centrifugation for 15 min at 4 °C [28]. The activity of superoxide dismutase (SOD) was estimated by its ability to inhibit epinephrine autoxidation [29]. Catalase activity (CAT) was measured as per Beutler [30], following the decomposition of H_2O_2. Glutathione S-transferase activity (GST) was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, according to Habig et al. [31]. Additionally, lipid peroxidation (LPO) was estimated in the livers through the formation of thiobarbituric reactive substances (TBARS) [32]. Briefly, solutions of trichloroacetic acid (TCA) 20% and butylhydroxytoluene (BHT) 4% were added to the samples and centrifuged (3200× *g*, 5 min). Then, thiobarbituric acid (TBA) 0.7% was added, and the samples were placed in a heat bath at 100 °C for 1 h and cooled before measuring. All determinations were performed in triplicate and expressed in terms of the sample protein content [33].

2.7. Short-Chain Fatty Acids (SCFA) and Lactic Acid Determinations

The determination of acetic acid, propionic acid, butyric acid, and lactic acid in the cecal content at the end of the treatment was carried out using high-performance liquid chromatography (HPLC), according to Ferrario et al. [34], with slight modifications. The samples were resuspended in H_2SO_4 0.01 M 1:10 (mobile phase) and centrifuged for 10 min at 10,000× *g*. The supernatant reached pH 2 with a fixed volume of H_2SO_4 2 M, and it was treated at 65 °C for 20 min. Another centrifugation step was carried out (16,000× *g*, 20 min, 10 °C), and the supernatant was filtered (0.45 µm membranes, Millipore, São Paulo, Brazil) before injection. The chromatographic system had two in-line detectors (Perkin Elmer, Norwalk, CT, USA): UV-visible (210 nm) and refractive index (200 Series and Flexar Series, respectively).

2.8. Statistical Analysis

A one-way ANOVA was used to compare independent treatments at a fixed time, and Kruskal–Wallis was performed when the assumptions for the ANOVA were not satisfied (Infostat, version 2020). Tukey and Dunn post-hoc tests were applied, respectively. Differences were considered significant at $p < 0.05$. A principal component analysis (PCA) was performed using the fviz_pca_biplot function from the factoextra R package (https://www.r-project.org/, accessed on 20 October 2023).

3. Results and Discussion

3.1. Microbiota Analysis

The barplot in Figure 1 shows the relative frequency for the bacterial groups that presented significant differences at the end of the treatment ($p < 0.05$), according to the Unifrac index. These taxonomic groups are listed in Table 1. On the other hand, no significant differences were observed among treatments, neither at the beginning nor the end of the assay for the Shannon index, used to estimate the alpha diversity.

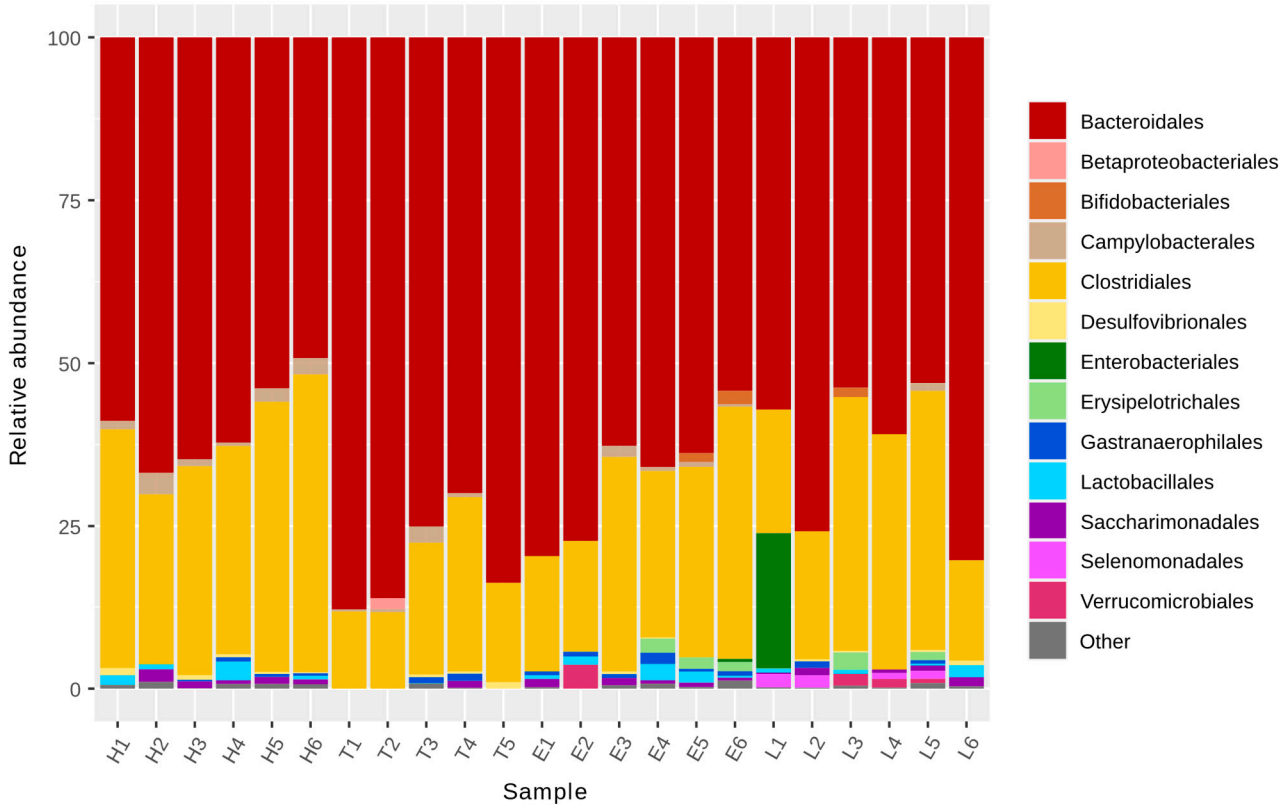

Figure 1. Bar plot representing the relative frequency of the bacterial orders that showed significant differences at the end of the treatment ($p < 0.05$). H: healthy group; T: TNBS-induced colitis group; E: TNBS-induced colitis group which received the purified EPS from Lf2; L: TNBS-induced colitis group which received Lf2.

The abundance of *Bacteroidaceae* was significantly increased in T (TNBS-treated mice) compared to group H, and it was slightly higher than the other treatments as well (T > L > E > H). In this direction, He et al. [35] studied the impact of TNBS-induced colitis in 7-week-old BALB/c mice, the same mouse strain used in this study. Animals were intrarectally administered ethanol (ET), TNBS in ethanol (TN), and phosphate-buffered saline solution as a control. The taxonomic structure of the mouse fecal samples showed that *Bacteroides* were significantly enriched in the TN group compared to the ET group, similar to our results. *Barnesiellaceae* was only found in L and E, the latter in lower abundance. The family *Helicobacteraceae* presented the lowest abundance in L, while C and E were similar, and H

showed the highest values. Alkadhi et al. [36] examined the appendiceal microbiome in a spontaneous colitic Winnie (Muc2−/−) mouse model. They described the presence of *Helicobacteraceae* only in colitic Winnie mice (but not control mice), in line with reports of increased *Helicobacter* in IBD patients. In this sense, Lf2 could reduce the abundance of this bacterial family, contributing to the balance of the intestinal microbiota. The family *Clostridiales* (vadin BB60 group) presented the highest values in E, followed by H, T, and L (in decreasing order). As far as we are concerned, there is no information regarding the role of the *Clostridiales* vadin BB60 group in colitis mouse models. However, decreased *Clostridiales* in mice have been related to more severe acute colitis, as well as decreased expression in butyrate synthesis genes [37]. Furthermore, Cheng et al. [38] found that the abundance of *Clostridiales* vadin BB60 was positively correlated with fecal SCFA in mice fed with a linear levan from a *Paenibacillus* isolate. *Veillonellaceae*, an acetate and propionate producer [39], was only found in L. Regarding *Lachnospiraceae*, this family had the highest abundance in H, followed by E, L, and T in decreasing order, with H, E, and L statistically similar. It includes known butyric acid producers, such as *Roseburia* and *Eubacterium* [40]. *Erysipelotrichaceae*, which had been positively associated with SCFA [41], was found in L and E only, and *Lactobacillaceae* showed the highest abundance in E, followed by H, L, and T (the latter being significantly lower than the other treatments). This family was associated not only with SCFA production but also with the activation of T-helper cells to resist foreign pathogens and regulate the secretion of cytokines [42,43]. Although higher levels for this family were expected for L, since it was the group that received the strain, it should be noticed that *Lactobacillaceae* includes numerous genera [44], making it difficult to assess the global impact of Lf2 by only comparing the levels of this family. Many of these bacterial genera could have been regulated and modified simultaneously, bringing about levels that do not necessarily represent the ones associated with the genus *Limosilactobacillus*. Moreover, the intestinal microbiota is a quite complex system in which different factors are involved in the overall response to a certain treatment. In this direction, the EPS could have promoted the increase in *Lactobacillaceae* by different metabolic pathways that might not have taken place during the administration of the strain itself. The order *Rhodospirillales* was found in E, followed by L, which was previously associated with the production of acetic acid, as this order includes bacteria from the genus *Acetobacter* [45].

Table 1. Absolute abundance of bacterial groups that showed significant differences among treatments after 15 days. Averages and *p*-values are provided. N = 6 for all treatments except for T (N = 5).

Phylum	Order	Family	H	T	E	L	*p*-Value
Bacteroidetes	*Bacteroidales*	*Bacteroidaceae*	220.33 ^{b}	457.60 ^{a}	318.17 ^{a}	383.67 ^{a}	0.012
Bacteroidetes	*Bacteroidales*	*Barnesiellaceae*	0.00 ^{b}	0.00 ^{b}	0.67 ^{b}	21.17 ^{a}	0.016
Epsilonbacteraeota	*Campylobacterales*	*Helicobacteraceae*	71.33 ^{a}	28.60 ^{b}	27.83 ^{b}	7.00 ^{c}	0.018
Firmicutes	*Clostridiales*	*Clostridiales*, vadin BB60 group	81.00 ^{b}	48.20 ^{b}	109.67 ^{a}	21.50 ^{b}	0.015
Firmicutes	*Selenomonadales*	*Veillonellaceae*	0.00 ^{b}	0.00 ^{b}	0.00	34.33 ^{a}	0.026
Firmicutes	*Clostridiales*	*Lachnospiraceae*	861.33 ^{a}	279.40 ^{b}	679.17 ^{a}	648.33 ^{a}	0.055
Firmicutes	*Erysipelotrichales*	*Erysipelotrichaceae*	0.00 ^{b}	0.00 ^{b}	51.00 ^{a}	30.50 ^{a}	0.074
Firmicutes	*Lactobacillales*	*Lactobacillaceae*	36.50 ^{a}	0.40 ^{b}	50.50 ^{a}	17.67 ^{a}	0.079
Proteobacteria	*Rhodospirillales*	Uncultured bacteria	0.00 ^{b}	0.00 ^{b}	13.17 ^{a}	0.83 ^{b}	0.014

H: Healthy; T: TNBS; E: EPS; L: *L. fermentum* Lf2. Different letters indicate significant differences among treatments ($p < 0.1$).

In a previous study, Jang et al. [46] studied the role of two strains of *L. fermentum* (KBL374 and KBL375, isolated from the feces of healthy Koreans) in mice with dextran sulfate sodium (DSS)-induced colitis. They found that the administration of these strains reshaped and increased the diversity of the gut microbiota. *L. fermentum* KBL375 favored beneficial microorganisms, such as *Lactobacillus* spp. and *Akkermansia* spp. Similarly, in our work, the L and E groups presented levels of *Lactobacillaceae* significantly close to the healthy control group (H) and higher than group T. Regarding *Akkermansia*, although the differences were not significant ($p > 0.05$), L and E presented higher abundance than T (18.7 and 30.2 vs. 0, respectively). In another work, Chou et al. [47] studied the effect

of a *L. fermentum* strain on azoxymethane/dextran sulfate sodium (AOM/DSS)-induced colitis-associated cancer. Sequencing data (16S rRNA) showed that this strain modified the composition of the gut microbiota by reducing the percentage of *Bacteroides.* The authors explained that the Gram-negative genus *Bacteroides* includes mucin-degrading bacteria with large endotoxins at their outer walls, resulting in mucosal damage and tumorigenesis [48]. Our results showed that the abundance of *Bacteroidaceae* was decreased in the L and E groups compared to T as well, but the differences were not significant.

In another work, Rodríguez-Nogales et al. [49] studied the effect of two probiotics (*L. fermentum* CECT5716 and *Ligilactobacillus salivarius* CECT5713) on a DSS colitic mouse model (male C57BL/6J) and found positive effects related to the amelioration of the microbiota dysbiosis and a recovery of the SCFA- and lactic acid-producing bacteria. In addition, *L. fermentum* restored the Treg cell population in mesenteric lymph nodes as well as the Th1/Th2 cytokine balance.

As regards the role of EPS in mouse models of colitis, there is scarce information as far as we are concerned. In this sense, this study is the first to associate the effects of the EPS-producing bacteria with its purified EPS. Although it is not possible to generalize about the effects of EPS from LAB on colitis, which depend on their chemical structure, molecular weight, and the conditions under which they were produced, among other factors, some authors suggest that they could be potential therapeutic alternatives to alleviate colitis [50,51].

3.2. Determination of Translocation, s-IgA, and Cytokines

Translocation of enterobacteria to the liver showed values <2 log(CFU/g), suggesting that Lf2 and its EPS might be safe to be used as food ingredients. However, further analysis is needed to confirm their safety. It is worth mentioning that, in preliminary studies, this strain proved to be sensitive to the antibiotics required by the European Food Safety Authority (EFSA) for this bacterial species [52], a concern that should always be addressed when studying probiotic microorganisms [53]. In the small intestine (Figure 2A), no significant differences were observed among treatments, except for IFN-γ which showed the lowest concentration in L ($p < 0.05$). On the other hand, the IgA levels in the intestinal fluid were significantly increased in E (Figure 2B). In the large intestine (Figure 2C), IL-10 showed similar levels among the treatments, while IL-12 presented lower values than the detection limit in L. The latter cytokine had similar values (>6000 pg g^{-1} tissue) in the other groups. The pro-inflammatory cytokines IFN-γ and IL-2 presented lower concentrations in L and E compared to H ($p < 0.05$) and T ($p > 0.05$).

Zhou et al. [54] studied the effect of *L. fermentum* CQPC04 on a DSS-induced colitis mouse model. They found that this strain increased colon length and ameliorated damage to the colon in colitic mice. In addition, it was able to inhibit the release of TNF-α, IFN-γ, IL-1β, IL-6, and IL-12 and increase the levels of IL-10 in serum. By RT-qPCR, the authors also confirmed that *L. fermentum* CQPC04 downregulated the expression of TNF-α, IFN-γ, IL-1β, and IL-6 and upregulated the expression of IL-10 in colon tissue. It seemed that the effect of this strain was exerted through the modulation of the NF-κB signaling pathway. These results suggested that the strain can reduce the phosphorylation of IκB-α, thereby preventing the activation of the NF-κB signaling pathway and ultimately reducing the release of other pro-inflammatory cytokines caused by NF-κB activation.

The same pathway was described for the strain *L. fermentum* HFY06 in a DSS-induced colitis mouse model [55] and for a recombinant strain (*L. fermentum* I5007) that expressed the *sodA* gene in a TNBS-induced colitis model, which inhibited NF-κB activity [56]. In addition, Li et al. [57] studied a synbiotic composed of arabinoxylan (AX) and *L. fermentum* HFY06 in a DSS-induced colitis model. Their results were in line with those previously described as the synergistic effect of AX, and the strain was associated with the inhibition of the NF-κB signaling pathway. Although it is not possible to confirm that the regulation of this pathway took place in our work, it seems plausible that Lf2 as well as its EPS could modulate the release of pro-inflammatory cytokines, especially in the large intestine.

Moreover, the EPS could enhance the production of IgA in the small intestine too, as observed in a previous study by our group performed on BALB/c mice that received EPS from Lf2 resuspended in yogurt [19].

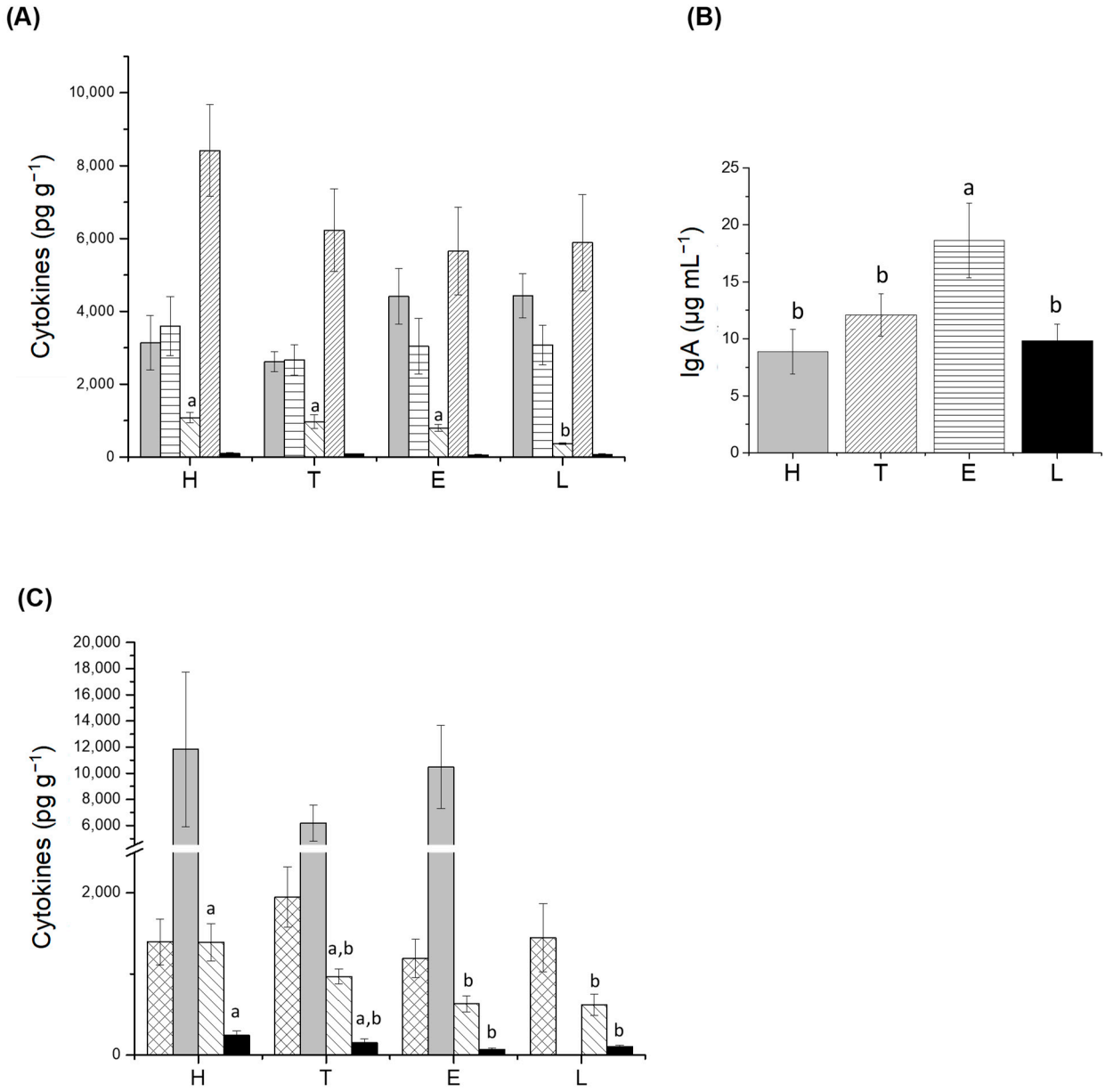

Figure 2. Immunomodulatory effects of Lf2 and its EPS in (**A**) small intestine: IL-12 (▭), TNF-α (▭), IFN-γ (▭), IL-6 (▭), IL-2 (■); (**B**) intestinal fluid; and (**C**) large intestine: IL-10 (▭), IL-12 (▭), IFN-γ (▭), IL-2 (■). H: healthy group; T: TNBS-induced colitis group; E: TNBS-induced colitis group which received the purified EPS from Lf2; L: TNBS-induced colitis group which received Lf2. Different letters indicate significant differences among treatments ($p < 0.05$).

3.3. Oxidative Stress

The imbalance between the production of reactive oxygen species and antioxidant systems is an important factor in the pathogenesis of colitis [58]. When inflammation takes place, local hypoxia leads to massive production of oxygen-free radicals, causing apoptosis or damage [59]. No significant differences were observed among treatments for GST and SOD enzymatic activities, while CAT presented the lowest activity in the TNBS-treated group (T) (Figure 3A). The treatment with the strain (L) presented values similar to those of the healthy group (H) ($p > 0.05$), while E showed increased activity compared to T but did

not reach the levels observed in H. Lipid peroxidation was not influenced by the treatments (Figure 3B). Our findings were consistent with the results obtained by Zhou et al. [54], who studied the effect of *L. fermentum* CQPC04 in a DSS-induced colitis model. The authors found that CAT activity was decreased in this colitis model and enhanced by treatment with the strain at high and low doses (1×10^{10} and 1.5×10^{9} CFU/kg of body weight, respectively), reaching values similar to the healthy control group. The authors also observed differences in the activity of SOD, but only with the high-dose treatment. In another study, Chauhan et al. [60] studied the antioxidant properties of *L. fermentum* Lf1 in a DSS colitis mouse model by RT-qPCR. A significant increase in the expression of antioxidant enzymes such as SOD2 and TrxR-1 was observed in the colitis-induced mice fed with the strain in comparison with the colitis-induced control. However, expression of the CAT gene could not be demonstrated since amplification could not be recorded for some groups.

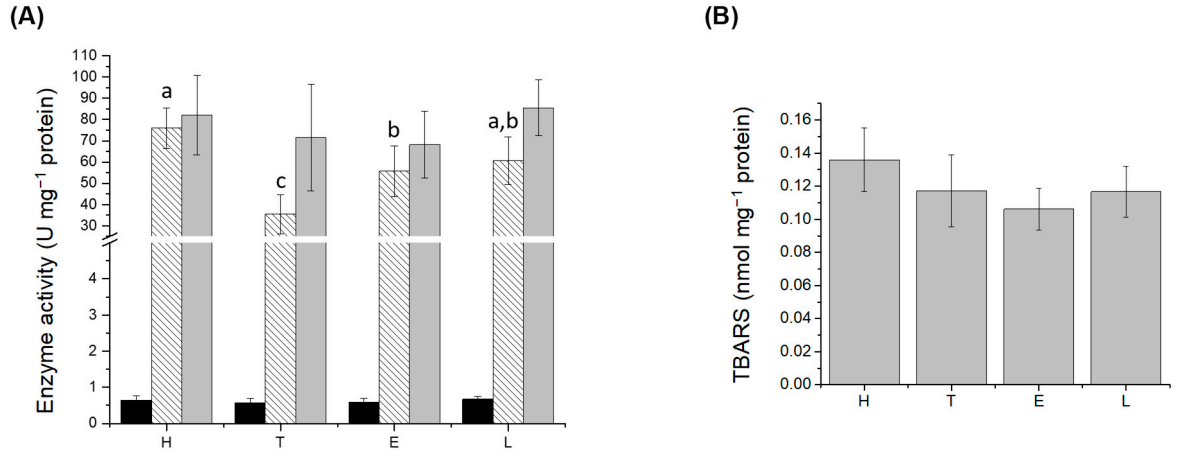

Figure 3. Antioxidant effects of Lf2 and its EPS in liver. (**A**) GST (■), CAT (▨), and SOD (▭) activities; (**B**) lipid peroxidation (LPO) levels in liver. H: healthy group; T: TNBS-induced colitis group; E: TNBS-induced colitis group which received the purified EPS from Lf2; L: TNBS-induced colitis group which received Lf2. Different letters indicate significant differences among treatments ($p < 0.05$).

3.4. Short-Chain Fatty Acids (SCFA) and Lactic Acid Determinations

The concentrations of propionic, butyric, and acetic acids were determined by the cecal content. Although lactic acid is not considered a SCFA, it was also quantified since it is one of the most important fermentation products of LAB. In addition, the fermentation products of some species can be incorporated as intermediate metabolites into the metabolic pathways of other species. For instance, pyruvate, lactate, and ethanol are diminished by bacterial utilization and SCFA synthesis [61]. The results showed that the group treated with Lf2 presented the highest levels of acetic and propionic acids (Figure 4), while the colitis-induced group T had the highest values of lactic acid ($p > 0.05$), suggesting that the latter acid must not have been turned into SCFA, at least to the same extent as the other groups. High concentrations of fecal lactic acid were previously reported in patients with ulcerative colitis and Crohn disease in comparison with a control group [62]. In this sense, Taylor et al. [63] reported that, during inflammation-associated dysbiosis (murine model), epithelial cells release lactate into the intestinal lumen, where it influences the metabolism of commensal gut microbes such as *E. coli* and *Salmonella*. In addition, the concentration of butyric acid in L reached levels similar to those observed in the healthy control group. Overall, it could be assumed that the treatment with the strain had a positive impact on the gut microbiota of mice with TNBS-induced colitis in terms of SCFA production. This result is in accordance with the modifications observed in the microbiota composition, as bacterial families related to the production of these metabolites, such as *Veillonellaceae*, *Lachnospiraceae*, *Lactobacillaceae*, and *Rhodospirillales*, showed higher abun-

dance in L. Although group E also presented an enhanced abundance of SCFA-producing families (*Lachnospiraceae*, *Lactobacillaceae*, and *Rhodospirillales*), this was not reflected in the concentration of these metabolites in the cecum content. Nevertheless, the EPS produced by Lf2 was previously demonstrated to have an important impact on the fecal SCFA when given to naïve BALB/c mice [20]. This ingredient, when added to yogurt exerted a prebiotic role, as observed in an increase in SCFA concentrations in feces at the end of the treatment, a property that was associated with an increase in the levels of *Clostridium* cluster XIVa, also known as the *C. coccoides* group, a well-known butyrate producer [64]. This difference could be attributed to the matrix in which the EPS was administered and the mouse model used, since in this previous study no challenge was performed.

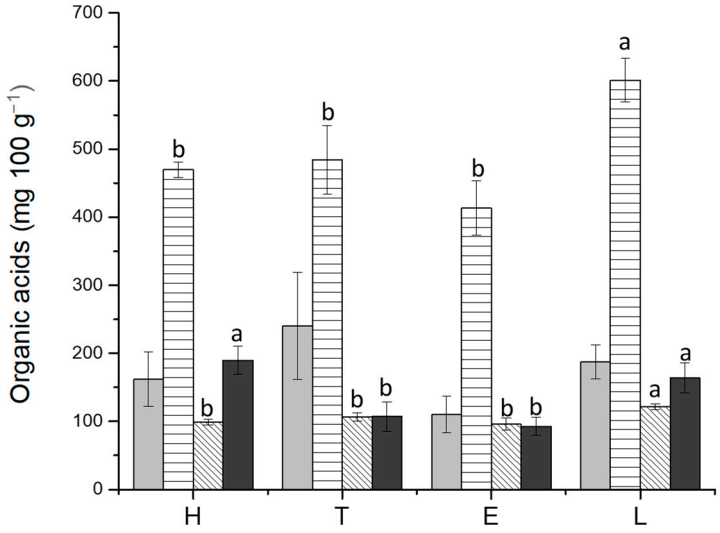

Figure 4. SCFA and lactic acid levels in cecal content: lactic acid (▭), acetic acid (▤), propionic acid (▨), and butyric acid (■). H: healthy group; T: TNBS-induced colitis group; E: TNBS-induced colitis group which received the purified EPS from Lf2; L: TNBS-induced colitis group which received Lf2. Different letters indicate significant differences among treatments ($p < 0.05$).

3.5. Multivariate Analyses

A multivariate analysis was performed to find statistically significant relationships among the parameters. In Figure 5, the PCA biplot can be observed for both PC1 vs. PC2 (Figure 5A) and PC1 vs. PC3 (Figure 5B), explaining 40.3% and 34.5% of the overall variance, respectively. Figure 5A clearly shows that H, E, and L, together with T, can be grouped in three different clusters. Treatment H was grouped on the positive side of PC1 and the positive side of PC2 and was associated with the levels of IFN-γ in both large and small intestines, IL-6 in the small intestine, CAT activity, IL-10 in the large intestine, TBARS, and *Helicobacteraceae*. The treatment E was grouped on the positive side of PC1 and the negative side of PC2, and it was associated with SCFA-producers such as *Lactobacillaceae*, *Rhodospirillales*, *Erysipelotrichaceae*, and *Clostridiales* vadin BB60. L and T were grouped on the negative side of PC1 and were associated with SCFA, lactic acid, *Veillonellaceae* (acetate producers), *Barnesiellaceae*, and *Bacteroidaceae*.

Figure 5B shows that T was separated from L by PC3. The latter is related to the three SCFAs—butyric; acetic; and propionic acids—and the families *Veillonellaceae*; *Barnesiellaceae*; and *Bacteroidaceae*. Furthermore, T was associated with lactic acid. This biplot grouped together E and H, which were related to *Lactobacillaceae*, *Rhodospirillales*, *Lachnospiraceae*, *Clostridiales* vadin BB60, GST, and CAT enzymatic activities, among other variables, including cytokines. From this analysis, it can be appreciated that both treatments E and L positively affected the immune profiles of TNBS-treated mice and were clearly separated from the colitis control group, especially in terms of SCFA levels in the cecum and gut microbiota composition. In a nutshell, Lf2 and its purified EPS could be potential functional food ingredients that might help to ameliorate or prevent the symptoms of chronic colitis.

In order to verify this hypothesis, clinical studies are required. The mechanisms by which they exert their overall effect are complex, as different factors converge, including the modulation of the immune system, the activity of antioxidant enzymes (CAT) in the liver, and gut microbiota homeostasis. In this particular case, the molecules that could play a crucial role in this response are EPS (purified or attached to the cell surface). For instance, a recent meta-analysis about the effects of these microbial metabolites on the immune system in animals with ulcerative colitis (UC) indicated that EPS could be an alternative or adjuvant treatment for UC, mainly regulating pro-inflammatory pathways [65]. In another work [50], in which the EPS from *Lacticaseibacillus rhamnosus* ZFM231 was studied in a DSS-induced colitis mouse model, similar results were obtained. The EPS-treated group recovered the diversity and composition of the gut microbiota to the levels of the normal group. The authors found that the EPS effectively modulated the gut microbiota, increased SCFA and TGF-β (anti-inflammatory cytokines), and decreased the levels of TNF-α. Nonetheless, more studies (preclinical and clinical) are required to disclose the connection between the properties of these molecules and the producing strain.

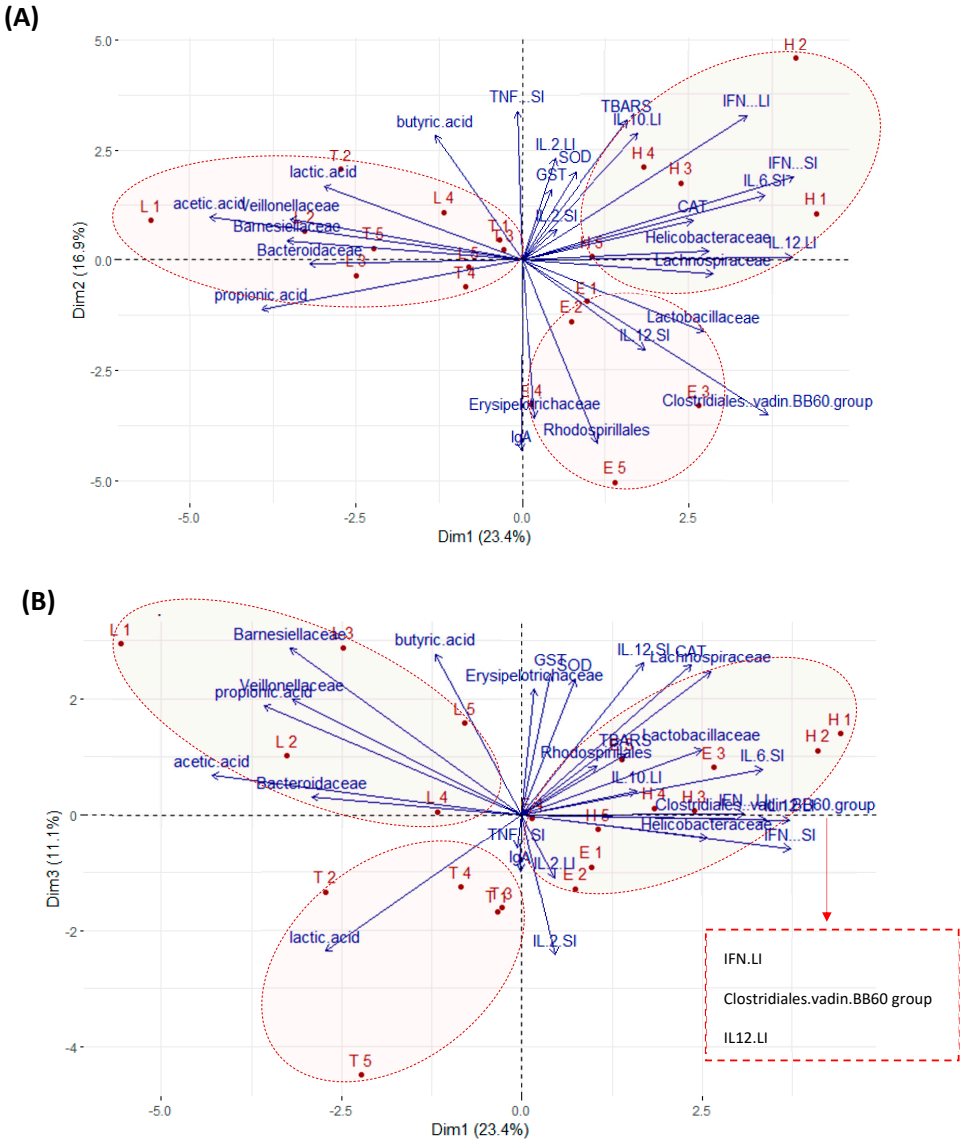

Figure 5. Principal component analyses including all the variables assessed. (**A**) PC2 vs. PC1; (**B**) PC3 vs. PC1. H: healthy group; T: TNBS-induced colitis group; E: TNBS-induced colitis group which received the purified EPS from Lf2; L: TNBS-induced colitis group which received Lf2; SI: small intestine; LI: large intestine.

4. Conclusions

This is one of the first studies to address the study of both a potentially probiotic strain (Lf2) and its purified EPS using a chronic TNBS-induced colitis model. The effects observed for the EPS-producing strain were similar to those of the purified ingredient in terms of immunomodulatory properties, antioxidant effects, and microbiota modulation, making it possible to suggest that the health-promoting activities of Lf2 could be related to its ability to produce these metabolites. To demonstrate this correlation, future studies could be conducted using the knockout strain lacking the genes involved in EPS synthesis. Although the effects might strongly depend on the mouse model used, food matrix, and concentrations chosen, among other factors, this study provides valuable insights into the protective role of EPS produced by LAB in health.

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Institutional Review Board Statement: This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of CONICET (CEYSTE-Comité de Ética y Seguridad del Trabajo Experimental, Argentina). This research did not involve human subjects.

Informed Consent Statement: Not applicable.

Data Availability Statement: All the raw data used in this study were deposited in the Sequence Read Archive as part of the bioproject PRJNA1063264. The data that support the findings are available from the corresponding author upon reasonable request.

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Conflicts of Interest: The authors declare no conflicts of interests.

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