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Functional properties of exopolysaccharide (EPS) extract from *Lactobacillus fermentum* Lf2 and its impact when combined with *Bifidobacterium animalis* INL1 in yoghurt

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ABSTRACT

The roles of an exopolysaccharide (EPS) extract from *Lactobacillus fermentum* Lf2 were studied individually or combined with a probiotic strain, *Bifidobacterium animalis* subsp. *lactis* INL1. EPS in its purified form caused an increase in the levels of cytokine TNF-α; both purified and crude EPS produced an increase in the regulatory cytokine IL-10. BALB/c mice received yoghurt with no additives (Y), with EPS (YE), with bifidobacteria (YB), or both (YEB) for 25 days. Only the YE group presented significantly increased concentrations of total short chain fatty acids ($p < 0.05$) including acetic and butyric acids; the levels of the *C. coccoides* cluster also rose over time ($p < 0.05$) for this group. A possible bifidogenic role was observed with the YEB group, reflected in the increasing levels of the genus *Bifidobacterium* along time ($p < 0.05$); this was not observed when the probiotic was administered solely (YB group).

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1. Introduction

Whereas probiotics are live microorganisms that when administered in adequate amounts confer a health benefit on the host (Hill et al., 2014), prebiotics are non-viable substrates that function as nutrients for beneficial microorganisms harboured by the host, including administered probiotic strains and indigenous microorganisms. Thus, a prebiotic should elicit a metabolism biased towards health-promoting microorganisms within the indigenous ecosystem. According to the consensus statement by ISSAP, the definition of a prebiotic has been recently modified to 'a substrate that is selectively utilized by host microorganisms providing a health benefit' (Gibson et al., 2017), including the impact of prebiotic at extraintestinal sites: on bone strength, neural and cognitive processes, immune functioning, skin, and serum lipid profile (Collins & Reid, 2016; Gibson et al., 2017). In addition, the term synbiotics refers to a combination of both, probiotics and prebiotics in a synergic way, so this term should be reserved for products in which the prebiotic compound(s) selectively favours the probiotic organism(s) (Cencic & Chingwaru, 2010).

The catabolism of prebiotic carbohydrates by the metabolic activity of the gut microbiota primarily produces three short chain fatty acids (SCFAs): acetate, propionate and butyrate. The most abundant SCFA in the colon is acetate and, in general, represents more than half of SCFA content detected in faeces (Louis, Scott, Duncan, & Flint, 2007). The prebiotic substrates are able to selectively promote the growth of beneficial microorganisms and induce changes in the levels of these acids in healthy individuals (Lecerf et al., 2012). Thus, the levels of these SCFAs represent an indirect measure of the level of beneficial microorganisms and their impact on human health.

Some lactic acid bacteria (LAB) are able to produce exopolysaccharide (EPS) as part of their metabolism; these polymers can be released to the medium exerting technological and functional roles. Regarding their functional properties, EPS from LAB have demonstrated several benefits including bifidogenic/prebiotic effects, immunomodulatory properties, prevention of pathogenic bacteria, gastritis, antitumor and antioxidant activities, among others (Ale et al., 2016a; Hamet, Medrano, Pérez, & Abraham, 2016; Polak-Berecka, Waśko, Szwajgier, & Chomaz, 2013; Rodríguez, Medici, Rodríguez, Mozzi, & Font de Valdez, 2009; Wang et al., 2014).

In this regard, Sarikaya, Aslim, and Yuksekdag (2017) reported that the lyophilised EPS from *Lactobacillus fermentum* LB-69 presented both bifidogenic and anti-biofilm effects (in vitro) against a strain of *Bacillus cereus*. Furthermore, *Lactobacillus rhamnosus* E/N is a probiotic strain that synthesises EPS with significant bifidogenic and antioxidant activities (Polak-Berecka et al., 2013). These positive aspects make EPS, as well as the producer strains, suitable ingredients for the formulation of novel functional foods.

In general, EPS are produced in situ during fermentation process of dairy food when, for example, EPS^+ starter or adjunct cultures are added to the food matrices to improve the textural and organoleptic characteristics of the final product (Amatayakul, Sherkat, & Shah, 2006; Hassan, Ipsen, Janzen, & Qvist, 2003). Only a few studies have described the effects of the direct application of bacterial EPS extracts as food additives. For example, a crude EPS from *Streptococcus thermophilus* suspended in milk was proposed to effectively prevent or heal chronic gastritis in a murine model (Rodríguez et al., 2009; Rodríguez, Medici, Mozzi, & de Valdez, 2010). So far, no report describes the application of EPS extracts as food ingredients in yoghurt, this option being an interesting

proposal in the case the EPS-producing strains are not suitable for growing in a food matrix.

L. fermentum Lf2 is an autochthonous strain that was isolated as non-starter culture from a local semi-hard cheese with blowing defects. This strain produces high amounts of EPS when it grows under controlled conditions of temperature (30 ºC) and pH (6.0), reaching 0.8 g L^{-1}, approximately, in semi-defined medium (SDM; Kimmel & Roberts, 1998) broth (Ale et al., 2016b). The total EPS is composed mainly of two polysaccharides: a β-glucan whose repeating unit is a trisaccharide (1.8×10^3 KDa), and a heteropolysaccharide constituting a disaccharide repeating unit of glucose and galactose, the main backbone being composed of α-(1→6) linked galactose residues, each one substituted by a terminal glucose residue (90 KDa) (unpublished data). From previous studies, this EPS extract presented not only interesting technological characteristics, such as an increase in consistency and pseudoplasticity of yoghurts (Ale et al., 2016b), but also positive health effects, protecting against a *Salmonella* infection and increasing the levels of IgA in intestinal fluid of mice (Ale et al., 2016a).

On the other hand, *Bifidobacterium animalis* subsp. *lactis* INL1 was isolated from breast milk and, as *L. fermentum* Lf2, belongs to the Instituto de Lactología Industrial (INLAIN) collection. This bifidobacterium has been widely studied regarding its technological and probiotic properties: resistance to storage in acidified milk, stability to spray-drying and freezing processes and IgA- mediated immunomodulation (Zacarías, Binetti, Laco, Reinheimer, & Vinderola, 2011), protection against acute and chronic colitis in spray dried form (Burns et al., 2017), and protection against *Salmonella* infection (Zacarías, Reinheimer, Forzani, Grangette, & Vinderola, 2014).

In the present work, the EPS extract from *L. fermentum* Lf2 was evaluated in vitro to preliminarily analyse its immunomodulatory role. In addition, the EPS was added to yoghurt, alone or combined with the probiotic strain *B. animalis* subsp. *lactis* INL1, to evaluate (in vivo assay) the effects on the gut microbiota (potential prebiotic effect) of the EPS or a synergic interaction (potential synbiotic effect) when they are administrated together. For this purpose, different bacterial groups were determined in faeces, as well as the levels of SCFA at different periods post treatment. IgA and cytokines levels were also measured in intestinal fluid and small intestine, respectively, and histological analyses were done at the end of the assay to evaluate if epithelial damage occurred in large and small intestines. The aim of this work was to develop a functional yoghurt with synbiotic properties.

2. Materials and methods

2.1. Organisms and growth conditions

L. fermentum Lf2 and *B. animalis* subsp. *lactis* INL1 (INLAIN collection) were stored at –80 °C in MRS (De Man, Rogosa and Sharpe, Biokar, Beauvais, France) broth plus 15% (v/v) glycerol. They were routinely grown in MRS broth at 37 °C for 16 h in aerobiosis and anaerobiosis (Anaeropack Anaero, Mitsubishi Gas Chemical Co., Inc., Germany) plus 0.1% (w/v) cysteine, respectively.

For yoghurt production, two commercial strains, *Streptococcus thermophilus* SC42 and *L. delbrueckii* subsp. *bulgaricus* 254 (both from Biochemical, Argentina), were selected based on their inability (visual test) to produce EPS in milk. They were routinely

grown in 10% (w/v) reconstituted skimmed milk (RSM) at 43 °C and stored at -80 °C in the same medium.

To design the calibration curves for qPCR analysis (Supplementary material, Table S1,), the following strains were used as well: *Bifidobacterium breve* 110 (INLAIN collection) and *Bifidobacterium bifidum* ATCC 35914 were cultured in the same way as *B. animalis* subsp. *lactis* INL1. *S. thermophilus* SC42 was grown in Elliker broth (Biokar) at 42 °C; *Escherichia coli* EC101 in LB (Luria-Bertani) broth, with continuous agitation, at 37 °C under aerobiosis and *Staphylococcus aureus* 76 (INLAIN collection) was developed in tryptic soy (TS) broth (Biokar) at 37 °C in aerobiosis. All cultures were grown from an isolated colony.

2.2. EPS production of L. fermentum *Lf2*

EPS production was carried out as described by Ale et al. (2016b). Briefly, cultivations were performed in a 2-L fermenter (Sartorius Biostat A plus®, Goettingen, Germany) in SDM (Kimmel & Roberts, 1998) broth with the aim to minimise interferences in EPS isolation by replacing yeast extract, beef extract and proteose peptone from MRS broth by yeast nitrogen base and Bacto Casitone (both from Difco, Becton, Dickinson and Company, Le Pont de Claix, France). *L. fermentum* Lf2 was inoculated from an overnight culture (0.1%, v/v) and incubations were made at 30 °C for 72 h, with agitation (6 rpm) and sparging with CO_2 (0.2 L min^{-1}). The pH was kept automatically at 6.0 with sterile 8 M NaOH. After incubation, bacteria were removed by centrifugation (19,630 × *g*, 30 min, 5 °C) and EPS was extracted and precipitated at 4 °C for 48 h by adding 2 volumes of chilled absolute ethanol (Cicarelli, Buenos Aires, Argentina). The precipitate was collected by

159 centrifugation (4,050 × *g*, 30 min, 5 °C), dissolved in ultrapure water and dialysed against
160 distilled water, using 12–14 kDa MWCO membranes (Sigma Aldrich, St. Louis, MO,
161 USA) for 3 days, at 4 °C with daily change of water. Finally, the EPS solution was freeze-
162 dried (Chris Alpha 1-4 LD Plus, Tokyo, Japan), weighed and expressed as mg crude EPS L^{-1}
163 . Additionally, a purification of the EPS crude fraction was performed with a treatment
164 with DNAse I (5 μg mL^{-1}; Sigma Aldrich) at 37 °C for 12 h and Pronase E (50 μg mL^{-1};
165 Roche, Germany) at 37 °C for 18 h. Then, a precipitation step with TCA (12%, w/v) with
166 subsequent neutralisation with NaOH was done. The suspension was dialysed against
167 distilled water and freeze-dried as indicated above to obtain the EPS purified fraction
168 (López et al., 2012).
169
170 *2.3. Preliminary in vitro assay to analyse the immunomodulatory role of EPS*
171
172 The THP-1 cell line was routinely grown in RPMI medium (Roswell Park Memorial
173 Institute medium, RPMI-1640 medium, Sigma Aldrich) containing 10% (w/v) of foetal
174 bovine serum and 50 mg mL^{-1} of streptomycin and penicillin. The cell line was incubated at
175 37 °C with 5% CO_2. The THP-1 monocytes were differentiated into macrophages by
176 incubation with phorbol 12-myristate 13-acetate 50 mM (PMA, Sigma Aldrich) for 48 h,
177 followed by an incubation for 24 h in RPMI medium. Macrophages derived from THP-1
178 were stimulated with crude or purified EPS: 60 μg mL^{-1} of the crude form containing 0.9%
179 proteins according to the Bradford method (Bio-Rad), and 12.6 μg mL^{-1} of the purified
180 form. This last concentration was proposed taking into account that, after purification of the
181 crude EPS extract, 21% (approximately) of purified EPS is recovered. This way both
182 samples contain approximately the same proportion of EPS. A positive control treated with

lipopolysaccharide (LPS, 0.5 μg mL^{-1}) was also included. The cells were incubated at 37 °C, with 5% CO_2 for 4 h for the detection of TNF-α, and 8 h for IL-6 and IL-10 determinations. The cytokine analysis was performed from the culture supernatants using the DuoSet ELISA kits (R&D Systems, Minneapolis, United States) according to the protocols recommended by the supplier. A negative control with untreated cells was also included. All determinations were made in quadruplicate.

2.4. In vivo assay to analyse the functional role of EPS as food ingredient in yoghurt

2.4.1. Manufacture of yoghurts

Yoghurts were made with 10% (w/v) RSM inoculated with *S. thermophilus* SC42 and *L. delbrueckii* subsp. *bulgaricus* 254 (10^6 and 10^5 cfu mL^{-1}, respectively), with 0 (control) and 600 (equivalent to 146 mg pure EPS L^{-1}) mg L^{-1} of crude EPS added. The incubation was at 43 °C until a final pH value of 4.6 was reached. The concentration of crude EPS was chosen according to a previous study in which the crude extract at 600 mg L^{-1} exerted protection against *Salmonella* infection (Ale et al., 2016a). After fermentation, yoghurts were immediately cooled and stored at 4 °C. The following day an overnight culture of *B. animalis* subsp. *lactis* INL1 was washed twice with sterile PBS and resuspended in yoghurt with 0 or 600 mg L^{-1} of EPS extract at a level of 5×10^8 cfu mL^{-1}; this way the final cell count was not reduced during yoghurt manufacture.

2.4.2. Animals and feeding procedures

For in vivo trials, 6-week old male BALB/c mice weighing 19–21 g were obtained from the random-bred colony of the Centro de Experimentaciones Biológicas y Bioterio

(FCV-UNL, Esperanza, Santa Fe, Argentina). Animals were humanly handled according to the National Institutes of Health guide for the care and use of Laboratory animals (NIH 8023, 1978). In addition, all the procedures were approved by the Ethical Committee for Animal Experimentation (FCV-UNL, Esperanza, Santa Fe, Argentina), and were made following the recommendations of the INLAIN animal facility (Zacarías et al., 2014).

Twenty eight mice were clustered in four groups (seven mice/group) and received, during 25 consecutive days and by gavage, 300 µL d^{-1} of any of the following samples: Y, yoghurt; YE, yoghurt + 600 mg EPS L^{-1} (equivalent to 9 mg kg^{-1} d^{-1}); YB, yoghurt + 5×10^8 cfu mL^{-1} *B. animalis* subsp. *lactis* INL1; YEB, yoghurt + 600 mg EPS L^{-1} + 5×10^8 cfu mL^{-1} *B. animalis* subsp. *lactis* INL1. All animals simultaneously and ad libitum received sterile tap water and a conventional balanced diet (Cooperación, Buenos Aires, Argentina). The diet composition was the following: 230 g kg^{-1} protein, 60 g kg^{-1} crude fibre, 100 g kg^{-1} total minerals, 13 g kg^{-1} Ca, 8 g kg^{-1} de P, 120 g kg^{-1} water and vitamins.

2.4.3. Bifidobacteria counts in faeces

Faeces samples were obtained at 0, 8, 18 and 25 days by the use of individual metabolic cages (Tecniplast, Buguggiate, Italy) for cell counts of total bifidobacteria, DNA extraction and SCFA quantification. Total bifidobacteria were determined on RCM agar (Reinforced Clostridial Medium, Biokar) with the selective supplement for bifidobacteria MUP (mupirocin, Merck, Darmstadt, Germany) according to Fanning et al. (2012), at initial time (before receiving treatment) and at 8, 18 and 25 days post-treatment. The selection of this medium was based on the supplementation with MUP, an antimicrobial compound that acts as a selective agent for the inhibition of anaerobic bacteria with the exception of bifidobacteria. The first dilution was done in sterile PBS buffer and the

231 mixture was placed in stomacher for 3 min at maximum speed. Successive dilutions were
232 done in 0.1% peptone water (w/v). The plates were incubated under anaerobiosis
233 (Anaerocult®, Merck) at 37 °C for 48 h and the morphology of the different colonies was
234 verified microscopically (1000×, phase contrast), selecting those typical of bifidobacteria.
235
236 *2.4.4. Estimation of the levels of different bacterial groups in faeces by qPCR*
237 Total DNA was extracted using the QIAmp DNA Stool Mini kit (Qiagen) from the
238 diluted faeces (1:10 in PBS) that were previously homogenised by stomacher (3 min at
239 maximum speed). Samples were kept at –20 °C until the moment of DNA extraction and
240 subsequent amplification. The selection of the microbial groups that were analysed was
241 based on in vivo studies carried out by reference groups using BALB/c mice (Gómez-
242 Gallego et al., 2012).
243 The quantitative PCR was done with a StepOnePlus thermocycler (Applied
244 Biosystems, Foster City, CA, USA), using the Power SYBR Green PCR Master Mix
245 (Applied Biosystems). The reaction cycles, for all cases, were: 95 °C during 10 min and 40
246 cycles of amplification (95 °C for 15 s, T° annealing for 1 min). The optimal annealing
247 temperatures for each reaction, the primers used, the efficiencies obtained in each
248 calibration curve, as well as and the standard used, are detailed in Supplementary material
249 Table S1. The R^2 values were higher than 0.994 in all cases.
250 DNA extractions from cultures were performed with the same kit used for mouse
251 faeces but from 5 mL of an overnight culture, to which a cell count was done on the same
252 day of the extraction. Three of the standard DNAs were purchased from the German culture
253 collection (DSMZ): DSM 17677 from *Clostridium leptum*, DSM 20438 from
254 *Bifidobacterium catenulatum*, DSM 935 from *Clostridium coccoides*, and for practical

255 purposes DNA was considered to come from an initial culture of 1×10^8 cfu mL^{-1}. All
256 cultures were grown from an isolated colony and all determinations were made, at least, in
257 duplicate for each sample. For primers corresponding to species *B. breve* and *B. bifidum*, as
258 well as for the genus *Staphylococcus*, values lower than the detection limit were obtained in
259 all samples analysed.
260
261 *2.4.5. Quantification of SCFAs in faeces*
262 The determination of SCFAs was carried out from faeces using HPLC at initial
263 time, 8, 18 and 25 days post-treatment, according to Ferrario et al. (2014) with some
264 modifications. The samples were diluted 1:10 in sterile PBS, treated in stomacher (3 min at
265 maximum speed) and centrifuged 10 min at 10,000 × *g*. Then the supernatant was acidified
266 with a fixed volume of concentrated H_2SO_4 for all samples until pH 2 was reached,
267 centrifuged as described previously, and finally filtered (45 μm, Millipore) before injecting
268 the samples to the equipment. The chromatographic system consisted of a quaternary pump,
269 an in-line degasser, a manual injector, an oven for temperature control of the column and
270 two in-line detectors: UV-visible (210 nm) and refractive index (Perkin Elmer). The data
271 were analysed and processed using the Chromera® software. An Aminex HPX-87H, 300 ×
272 7.8 mm column, an Aminex Cation-H column guard (30 × 4.6 mm) (Bio-Rad Laboratories)
273 and an isocratic flow of 0.6 mL min^{-1} with a mobile phase of H_2SO_4 0.01 M were used, at a
274 temperature of 65 °C. The identification was made with the UV and IR detectors comparing
275 the retention times of standard solutions (Sigma Aldrich), while the quantification was
276 carried out by means of the refractive index detector, since the chromatograms obtained
277 were cleaner. Three replicates of each sample were done.
278

279 **2**.*4.6. Determination of s-IgA and cytokines in small intestine*

280 After the feeding period, animals were injected intraperitoneally with an anaesthetic
281 cocktail prepared with 1.8 mL of ketamine (50 mg mL^{-1}, KetonalTM, Richmond Vet
282 Pharna, Argentina), 0.9 mL of xylazine 2% (Alfasan, Argentina), 0.3 mL of acepromazine
283 (10 mg mL^{-1}, Acedan, Hollyday, Argentina) to a final volume of 10 mL, adding 7 mL of
284 sterile saline solution. It was kept at 4 °C until the moment of application (0.3 mL per
285 mouse). Mice were then sacrificed by cervical dislocation. Liver was removed and
286 homogenised in 5 mL sterile PBS and pour plated onto ABRV agar (37 °C for 24 h in
287 aerobiosis) to evaluate translocation of enterobacteria to liver. s-IgA and cytokines were
288 determined as described previously by Ale et al. (2016a). s-IgA was determined in
289 intestinal fluid and cytokines IL-10, IL-6, TNF-α and IFN-γ were determined in the distal
290 small intestine tissue (jejunum and ileum), using the corresponding mouse ELISA Set (BD
291 OptEIA, BD, Biosciences PharMingen, San Diego, CA, USA).
292
293 *2.4.7. Histological analysis*

294 Histological analysis was done as described by Burns et al. (2017). Briefly, it
295 consisted of placing the tissues in cassettes for histology and submerging them overnight in
296 a solution of formaldehyde (4%, v/v, in PBS; Ciccarelli, Buenos Aires, Argentina). The
297 next day, the tissues were dehydrated by successive passages in solutions with increasing
298 concentrations of ethyl alcohol. For rinsing, the cassettes were allowed to dry on absorbent
299 paper and treated with toluene for 30 min. Once dried, the samples were embedded in
300 paraffin and the sections were kept at 4 °C. For the histological analysis, 5 μm sections
301 were made and stained with May Gründwald (MG)-Giemsa. The dye MG was diluted 1: 5
302 and the Giemsa 1: 100 in distilled water. The paraffin was removed and the sections were

rehydrated in successive baths of toluene, ethyl alcohol and, finally, distilled water. The coloration consisted in leaving the section 15 min in MG (37 °C), 40 min in Giemsa (37 °C) and two subsequent baths in distilled water. For the differentiation, the samples were placed 30 s in acetic acid (dilution 1: 100) and 10 s in alcohol/acetone (50: 50). Finally, the assembly was carried out, leaving the sections in toluene for a few seconds. A drop of EUKITT® (Sigma Aldrich) was placed on each slide and they were allowed to dry at room temperature. At least two sections were analysed per animal.

2.5. Statistical analysis

For statistical analysis, SPSS software (SPSS Inc., Chicago, IL, USA) was used. ANOVA or Kruskal-Wallis was applied if the ANOVA assumptions were not satisfied, to analyse the treatment factor at a fixed time. The differences between means were determined by the Tukey test or by Dunns, respectively. Repeated measures test was applied to analyse the factor time. Sphericity criteria was always verified by the Mauchly test, and the multivariate statistics were considered in the case this criterium was not satisfied. Multiple comparisons were made with Bonferroni. The differences were considered significant when $p < 0.05$ for all the tests described above. In addition, a principal components analysis was included for SCFAs and bacterial groups, for which the Minitab 16 statistical program (Minitab Inc., State College, PA, USA) was applied.

3. Results

3.1. Preliminary in vitro assay to analyse the immunomodulatory role of EPS

Table 1 shows the results obtained for the quantification of TNF-α, IL-6 and IL-10 cytokines in the supernatant of the THP-1cells treated with purified and crude EPS, at a concentration of 12.6 μg mL^{-1} and 60 μg mL^{-1}, respectively. Simultaneous comparisons were done between all groups: cells treated with crude or purified EPS, LPS or untreated cells. Regarding TNF-α cytokine, significant differences ($p < 0.05$) were observed between cells treated with LPS (lipopolysaccharide, positive control) and those untreated, as expected, and between purified EPS and control without treatment. No significant differences were detected for cell treated with crude EPS extract. On the other hand, EPS in either form, purified or crude, seem not to have affected the levels of cytokine IL-6. For the cytokine IL-10, there were no significant differences between the cells treated with pure and crude EPS, while the levels of cells treated with LPS or untreated could not be detected.

3.2. In vivo assay to analyse the functional role of EPS as a food ingredient in yoghurt

The chosen dose for EPS extract was appropriate to address the experimental in vivo design, since no translocation was observed in mice from different groups, supporting the safety of the EPS and the probiotic strain (or their combination) at the proposed dose for oral consumption.

3.2.1. Bifidobacteria counts in faeces

Fig. 1 shows the results for bifidobacteria counts at 8, 18 and 25 days of treatment, subtracting the initial level of bifidobacteria from each mouse, in logarithmic scale. After 8

days of treatment, significant differences were found between YE and YB groups, the latter presenting approximately one more order than the first. At 18 days no significant differences were seen between groups, probably due to the high variability obtained, and, at the end of the treatment. No group differed from the control group.

The reason for the great variability observed (mainly at 18 days of treatment) remains unknown, but it could be due to intrinsic variability among mice population, as well as to the methodology employed for bacterial count. Although cell morphology was checked for all colonies, possibly some of them were not effectively bifidobacteria. DNA sequencing of each colony would have been needed to confirm its nature, but it was beyond the scope of this work.

3.2.2. Estimation of the levels of different bacterial groups in faeces by qPCR

The universal primers were designed to have specificity with the conserved regions of the rRNA-16S of prokaryotic cells (Baker, Smith, & Cowan, 2003), so they are related to the total microbial load present in the intestine. In this case, no differences were observed between groups at each time evaluated, but a significant effect was observed for the control group (Y) during the time of treatment (Fig. 2A). At 25 days the levels of total bacteria were significantly lower ($p < 0.05$) than those observed at 8 and 18 days. For the remaining groups there were no significant differences throughout time.

When the levels of the genus *Bifidobacterium* were estimated, significant differences were detected for the factors time and treatment (Fig. 2B). Differences among treatments were observed only at 25 days for the group YEB, which presented values significantly higher than the group YE ($p < 0.05$), but none differed from the control group (Y) and group YB. This was also confirmed by the cell count estimations, since at 25 days,

the group YEB presented higher levels of this genus in comparison with the group YE (Fig. 1). When evaluating the influence of time on each treatment, the control group (Y) and the group that received only EPS (YE) presented levels significantly lower at 18 days than those obtained at 25 days, indicating that the population of bifidobacteria increased towards the end of the assay. No significant differences were observed during time for YB treatment, while the levels obtained after 25 days were higher than those observed at 8 and 18 days for YEB group ($p < 0.05$). Thus, considering both factors, the results suggest that the combination of the two ingredients, EPS and the bifidobacteria, would play a possible synergic role, since the levels of the genus *Bifidobacterium* at the end of the treatment remained higher than those obtained at 8 and 18 days. It should be highlighted that, for the statistical analysis of the design applied, when the presence of repeated measures over time is considered as another source of variability, a better estimation of the error is produced.

Estimations of the levels of the species *B. catenulatum* (Fig. 2C) in the mouse faeces were also done. As the interaction between time and treatment factors was significant, it was not possible to analyse them separately. When this occurred, ANOVA (or Kruskal-Wallis) was applied at fixed time points, since the treatment groups were independent. At 8 and 25 days no significant differences were observed between treatments, but at 18 days significant differences were observed among the groups Y and YE, presenting the latter minor levels. Apparently, neither the EPS extract nor the bifidobacteria would stimulate the development of this bacterial species in the intestinal tract of the animals.

The levels of the *B. animalis* species were also determined by qPCR and the interaction between factors was again significant ($p < 0.05$). Differences were observed at all times evaluated and a general tendency to increase levels towards the end of the assay

was appreciated (Fig. 2D). After 8 days, the groups that received the bifidobacteria (YB and YEB) presented significantly higher levels than the control group (Y). Although this increment could be associated to the administration of the probiotic strain, it is interesting to evaluate the estimations after 18 days. In this case, the only group that presented significant differences in comparison with the control group was YEB, indicating that the combination of EPS and bifidobacteria was more effective in increasing the levels of the species *B. animalis* at this time. At 25 days, only significant differences were observed ($p < 0.05$) between groups YEB and YE, but none was different from the control group.

Regarding the *C. coccoides* group, time and treatment factors presented significant differences (Fig. 2E). YE group showed significantly lower levels than YEB group at 8 days, but both were similar to the control group. When analysing the effect of time for each treatment, differences were detected ($p < 0.05$) between days 18 and 25 in comparison with day 8 for group YE, indicating a significant increase of this bacterial group which remained high towards the end of the treatment. For the group YEB, differences were seen between days 18 and 8, while time did not have a significant effect on the other treatments. This information suggests that EPS could be responsible for the increase in the levels of this bacterial group over time, being more effective after 18 days of administration, and this effect seems to be more important when EPS was combined with the probiotic strain at 18 days of treatment.

For the genera *Streptococcus* (Fig. 2F) and *Lactobacillus* (Fig. 2G) and the group of *C. leptum* (data not shown), no significant differences were observed among treatments and control group.

3.2.3. Quantification of SCFAs in faeces

Fig. 3A shows the results obtained for lactic acid; although it is not considered a SCFA, its levels are related to the metabolism of LAB and other intestinal bacteria. Also, it is feasible to be converted to butyrate and propionate by the gut microbiota (Bourriaud et al., 2005). In our study, after 8 days, group YB showed significantly higher concentrations of lactic acid than YE, but none was different from the control group. At 18 days no differences were observed between treatments, while at 25 days the control group Y presented higher lactic acid levels ($p < 0.05$) than the groups that received the bifidobacteria, either alone or together with the EPS. These results suggest that, despite the fact that the group YB reached high levels of lactic acid at 8 days, these levels were significantly lower at the end of treatment in comparison with the control group, either in combination or not with the EPS of *L. fermentum* Lf2.

Regarding acetic acid (Fig. 3B), the group YE presented levels significantly higher at the end of the treatment in comparison with 8 days. For the other treatments, no significant changes were observed during the 25 days of intervention, or among the four groups at each time analysed. The same observations can be made for the results obtained for butyric acid and the sum of the three SCFAs (Fig. 3C and 3D, respectively), showing an increment in the levels of both acids for group YE. For propionic acid, despite presenting a similar behavior, no significant differences were observed among groups or during treatment (data not shown).

These results indicate that the treatment that favours the production of acetic acid, butyric acid and the sum of the three SCFAs is YE. In the case of the combination of both ingredients (group YEB), no significant differences were observed during treatment. Considering these results, it seems that EPS alone is able to exert a clear effect on the production of SCFA, effect that was not detected when combined with the bifidobacteria.

447 The analysis of principal components (Fig. 4) was included to summarise the results
448 for the levels of some bacterial groups studied (eight were included in this analysis, those
449 which presented more influence) and the levels of the four organic acids at 25 days of
450 treatment. From Figs. 4A and 4B, which show the score and loading graphs for the first two
451 principal components (PC), respectively, it can be observed that all the variables of the
452 bacterial groups impact mainly on the PC1, while, on the other hand, an influence mainly of
453 organic acids is observed on the PC2. It could be appreciated that samples YEB and YB
454 were grouped in the negative hemiplane of PC1, characterised by the variables
455 *Bifidobacterium*, *Streptococcus*, *B. animalis*, *B. catenulatum* and *C. coccoides*.
456 In general, samples YEB were grouped at a greater distance than YB from the
457 origin, indicating that these variables have a greater effect on the YEB samples. The control
458 samples (Y) were mainly grouped in the positive hemiplane of PC2, characterised by the
459 variables lactic acid, enterobacteria and *Lactobacillus*. On the other hand, YE samples were
460 grouped, together with YE and YB, in the negative hemiplane of PC2, characterised by
461 butyric, acetic and propionic acids. It can be observed that the treatment with EPS (YE)
462 caused a greater impact on the concentration of the SCFA in the faeces of the treated
463 animals, while the treatment with the combination of both ingredients (YEB) had a more
464 marked influence on the population of bifidobacteria mainly, and on the genus
465 *Streptococcus* and the cluster *C. coccoides*, at 25 days. The group YB presented, on the
466 other hand, an intermediate behaviour, located in the centre of the plane.
467
468 *3.2.4. Determination of s-IgA and cytokines in small intestine*
469 Quantification of the level of different cytokines (IL-10, IL-6, IFN-γ and TNF-α) in
470 small intestine tissue is shown in Fig. 5A. Although the two groups that received EPS

extract (YE and YEB) presented higher values of the regulatory cytokine IL-10 than the other groups, no significant differences were observed for all the cytokines evaluated. Fig. 5B shows s-IgA concentrations in intestinal fluid and no significant differences were observed between treatments, probably because the maximum peak occurred before 25 days. This could be justified considering that a significant increase in s-IgA concentration was observed at 15 days of treatment, when the EPS was added to yoghurt at half the concentration used in the present assay (Ale et al., 2016a).

3.2.5. Histological analysis

The applied treatments caused no damage to the intestinal mucosa since normal morphology for both the small (Fig. 6) and large intestines (data not shown) of all the mice was observed. None of the tissues exhibited signs of inflammation or lymphocytic infiltration towards the mucosa and submucosa.

4. Discussion

By in vitro analysis, we could demonstrate that EPS from *L. fermentum* Lf2, in its purified form, caused an increase in the levels of the proinflammatory cytokine TNF-α, while both forms of EPS (purified and crude) produced an increase in the regulatory cytokine IL-10. As TNF-α is an important proinflammatory cytokine, a stimulation of its levels represents a typical immune tolerant phenotype. Both TNF-α and IL-6 play an important role in the signalling system for the initiation of the mucosal inflammatory response when the host intestinal epithelial surface is invaded by microbial pathogens (Jung et al., 1995). In addition, IL-10 is essential for maintaining the integrity and homeostasis of

the epithelial layers, limiting the damage caused by viral and bacterial infections through the repression of proinflammatory responses that could led to unnecessary tissue disruptions (Iyer & Cheng, 2012). Thus, TNF-α and IL-10 have potential implications given its central role in the inflammatory bowel diseases, both Crohn's disease and ulcerative colitis (Papadakis & Targan, 2000).

Gao et al. (2017) have reported changes in a number of proinflammatory cytokines (IL-12, IL-6 and TNF-α) with pre-treatment with EPS from *L. rhamnosus* GG in combination with LPS stimulation when compared with the LPS stimulation alone. López et al. (2012) studied the cytokines produced in peripheral blood mononuclear cells treated with EPS purified from different bifidobacteria. They did not detect any significant differences in the levels of IL-1β, IL-17 and IL-8 either with the presence of purified EPS or the EPS producing strains with respect to the control. In this case, the concentration of IL-10 was not modified with the EPS but it was increased with the producing bacteria, while the concentrations of IFN-γ and TNF-α were increased with both EPS and their producing bacteria In general, they observed that neutral and high molecular mass ($> 10^3$ kDa; Vaningelgem et al., 2004) EPS were not efficient to induce the immune response, while those with low molecular mass ($<10^3$ kDa) and negative charge triggered a stronger response.

Considering the chemical and structural characteristics of the *L. fermentum* Lf2 EPS extract (two uncharged fractions of low and high average molecular mass, unpublished data), it seems that the low molecular mass polysaccharide could have directed the response obtained, since it is present in a higher proportion than the high molecular mass polysaccharide (unpublished data). According to our results, the purified EPS from *L. fermentum* Lf2, at a single concentration studied (12.6 μg mL^{-1}) was able to cause a

519 stronger immune response than the crude extract, stimulating the production of the
520 cytokines TNF-α and IL- 10, while crude EPS (60 μg mL^{-1}), induced only the production of
521 IL-10. In view of this information, the purified form triggers a stronger immune response
522 towards a proinflammatory profile when the concentrations between the cytokines TNF-α
523 and IL-10 are compared (433 versus 28 pg mL^{-1}). In contrast, crude EPS only significantly
524 stimulates the production of IL-10 (37 pg mL^{-1}) indicating that, possibly, there may be
525 some components of unknown nature (maybe peptides or proteins) in the crude extract that
526 inhibit the production of this proinflammatory cytokine. As described by Peña and
527 Versalovic (2003), intestinal lactobacilli can produce soluble protein factors that
528 presumably bind to cell surface receptors and inhibit synthesis or secretion of TNF-α.
529 These authors observed that, in the case of *L. rhamnosus* GG, a decrease of TNF-α
530 production in LPS-activated murine macrophages took place by a contact-independent
531 manner. Further investigations should be carried out to elucidate the nature of the
532 components present in the EPS crude extract and to probe the mechanisms of these effects
533 and their in vivo relevance.

534 Moreover, it is well known that gut microbiota dysbiosis impact in the development
535 of diverse chronic disorders such as inflammatory bowel disease and, therefore, is a key
536 target for the intervention with functional ingredients (probiotics and/or prebiotics), which
537 could modify the altered gut microbiota, restoring the health state (Burns et al., 2017). In
538 this scenario, EPS with functional properties demonstrated and administered as food
539 ingredients (as in this case), could function as active players. For this reason, we decided to
540 study the modifications of the mice microbiota caused by the administration of the EPS of
541 *L. fermentum* Lf2 in a dairy matrix, alone or combined with a probiotic bifidobacterium.
542 Although the faecal microbiota does not strictly reflect the entire gastrointestinal

ecosystem, the determination of bacterial levels in faeces can be considered an acceptable approximation of the bacterial content of the distal colon (Hamet et al., 2016). From the analysis of the universal primers, it could be suggested that the administration of EPS and *B. animalis* subsp. *lactis* INL1, separately or together, would prevent the decrease of the microbial load over time, when compared with the group that received only yoghurt.

When the levels of the genus *Bifidobacterium* were estimated by cell counts, although it is more difficult to observe a clear tendency due to the variability of the results among animals, at day 25 the difference detected seems to obey exclusively to the presence of the bifidobacterium strain added. In this case, it would be more appropriate to considerer the results of qPCR determination for this bacterial genus due to the high specificity of the method. The qPCR results indicated that the combination of EPS and the probiotic strain (group YEB) seems to be effective in increasing the levels of the total *Bifidobacterium* during all the evaluated period (difference of 0.7 log cfu g^{-1}), approximately, between 8 and 25 days of treatment) when compared with the group of animals that received exclusively *B. animalis* INL1 (group YB; difference of 0.4 log cfu g^{-1}), indicating that the combination of both ingredients exerted a possible bifidogenic role. Salazar, Gueimonde, Hernández-Barranco, Ruas-Madiedo, and de Los Reyes-Gavilán (2008) observed, by batch cultures of faecal samples, that 11 different bifidobacterial EPS had a bifidogenic effect, detecting (PCR-DGGE) changes of other microbial groups during fermentation, mainly of *Bacteroides*, *E. coli* and microorganisms related to the second. Hamet et al. (2016) showed by DGGE that, when kefiran was orally administered to BALB/c mice, it modified the intestinal and faecal microbiota, increasing the population of bifidobacteria. Sarikaya et al. (2017) found that the lyophilised EPS of *L. fermentum* LB-69, at a concentration of 1 mg

566 mL^{-1}, exerted a bifidogenic effect on *B. breve* BASO-1 strain and prevented the formation
567 of biofilms by pathogenic bacteria.

568 In our case, although at 25 days of treatment the group YEB showed significant
569 differences only with group YE, we consider that, since the repeated measures test presents
570 a better estimation of the error than ANOVA, it would be also valid to evaluate the
571 evolution along time for each group. For the bifidobacteria species analysed, *B.*
572 *catenulatum* and *B. animalis*, no significant changes during time of treatment could be
573 evaluated due to the significant interaction among factors. But, when comparison among
574 treatments was considered, for the first one a lower level ($p < 0.05$) was observed at 18
575 days for mice that received EPS (YE) with respect to the control group, but this difference
576 was not appreciated at the end of treatment. In the case of *B. animalis*, the groups that
577 received the bifidobacteria with EPS (YEB) or alone (YB), presented significantly higher
578 levels than the control group at 8 days of treatment, which remained significant only for the
579 group YEB at 18 days. At the end of treatment, all groups were statistically similar to the
580 control group. These results suggest that the administration of EPS could have helped to
581 maintain the levels of this species different from the control group for a longer time of
582 treatment.

583 SCFAs are volatile fatty acids produced by the gut microbiota in the large bowel as
584 fermentation products from food components that are unabsorbed/undigested in the small
585 intestine, having distinct physiological effects. It has been shown that bifidobacteria can
586 protect the host against enteropathogenic infections through the production of acetate
587 (Fukuda et al., 2011). Acetate has been related to appetite reduction through its interaction
588 with the central nervous system (Frost et al., 2014) and the three SCFAs have been
589 associated with intestinal anti-inflammatory properties (Tedelind, Westberg, Kjerrulf, &

590 Vidal, 2007). Butyrate participates in the motility of the colon, reduces inflammation,
591 increases visceral irrigation, induces apoptosis and inhibits the progression of tumour cells,
592 properties that contribute with the prevention of colorectal cancer (Canani, 2011; Zhang et
593 al., 2010).

594 From qPCR quantifications, the levels of *C. coccoides* (group that belong to the
595 *Clostridium* cluster XIVa), were favoured over time for EPS consumption, either
596 individually or in combination with the bifidobacterium. This group has been described as
597 an essential component of the human gut microbiota (Duncan, Barcenilla, Stewart, Pryde,
598 & Flint, 2002), being responsible for the synthesis of large amounts of butyrate that is not
599 only used as the main energy source of the colon epithelial cells (Barcenilla et al., 2000;
600 Duncan et al., 2002), but also inhibits the expression of proinflammatory cytokine mRNA
601 in the mucosa (Segain et al., 2000). In addition, the decrease in this group has been related
602 to a higher incidence of Crohn's disease (Manichanh et al., 2006). Considering this
603 information, the increase in the final concentration of butyric acid in faeces for the group
604 YE could be probably associated to the increase observed for the *Clostridium coccoides*
605 cluster. These results suggest a positive impact on health, evidencing a functional role of
606 the EPS produced by *L. fermentum* Lf2. In a review by Besten et al. (2013), the relationship
607 between SCFA and intestinal microbiota was described. According to this work, the
608 phylum *Bacteroidetes* would be related to the production of acetate and propionate, while
609 the phylum *Firmicutes* (including *Clostridium* genera) would be more linked to the
610 synthesis of butyrate, reinforcing the observations of our study. The results regarding the
611 qPCR determinations, as well as the SCFA levels, were summarised in the biplot (PCA
612 analysis), which indicated that an increase in the levels of *Bifidobacterium*, *Streptococcus*,

613 *B. animalis*, *B. catenulatum* and *C. coccoides* can be related to the YEB group, while
614 increased levels of SCFA can be related to the group that received EPS solely (YE).
615 When the role of EPS on immunomodulation was analysed by determining the
616 levels of s-IgA and cytokines in small intestine, no effect was evidenced at 25 days of
617 treatment. Although in a previous report an immunomodulatory effect (increase on the level
618 of s-IgA) was observed for this EPS extract after 15 days of administration (Ale et al.,
619 2016a), a consequence of the prolonged administration could be assumed in the present
620 study. The observations by other authors (Moreno de LeBlanc et al., 2008) could justify our
621 results.
622

623 **5. Conclusion**

624

625 We demonstrated that, when added as a food ingredient in yoghurts, the EPS crude
626 extract from *L. fermentum* Lf2 exerted a possible prebiotic role reflected in an increase in
627 SCFA levels in faeces towards the end of the treatment, which could be explained by an
628 increase in the levels of the bacterial groups that are known to produce these beneficial
629 acids for health (cluster XIVa of *Clostridium*, also known as *C. coccoides* group). When
630 this EPS was combined with the probiotic strain *B. animalis* subsp. *lactis* INL1, a
631 bifidogenic effect throughout the time of treatment was appreciated, improving the effect
632 observed for both individual ingredients, fact that could be associated with a possible
633 synergism between them, suggesting a possible synbiotic role. Although it would be
634 necessary to demonstrate the effective health benefit associated to the recent definition of
635 prebiotic, considering our results, together with previous studies referring to other
636 functional (protection against *Salmonella* infection and immunomodulation) and

637 technological properties, the application of the EPS crude extract from *L. fermentum* Lf2 as
638 a potential techno-functional ingredient can be proposed for the design of novel foods.
639
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648
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Figure legends

Fig. 1. Bifidobacteria counts in faeces. Cell counts at initial time (t_0) were subtracted from those obtained at 8, 18 and 25 days for each mouse, in logarithmic scale. $\bar{x}$ ± SEM (vertical bars) is presented for each group at different times: ○, YE (group treated with crude EPS); ▼, YB (group treated with *B. animalis* subsp. *lactis* INL1); ◆, YEB (group treated with both ingredients); ●, Y (control group). Different letters indicate significant differences among groups at a fixed time point; determinations were done at least in duplicate.

Fig. 2. Levels of different bacterial groups in faeces by qPCR: A, universal primers; B, *Bifidobacterium*; C, *B. catenulatum*; D, *B. animalis*; E, *Clostridium coccoides*; F, *Streptococcus*; G, *Lactobacillus*. $\bar{x}$ ± SEM (vertical bars) is presented for each group at different times: ○, YE (group treated with crude EPS); ▼, YB (group treated with *B. animalis* subsp. *lactis* INL1); ◆, YEB (group treated with both ingredients); ●, Y (control group). Different letters indicate significant differences among groups at a fixed time point, while different number of asterisks indicates significant differences throughout time of treatment for each group separately; determinations were done in triplicate. Levels at t_0 were subtracted for each mouse, in logarithmic scale.

Fig. 3. Concentrations of different organic acids in faeces by HPLC: A, lactic acid; B, acetic acid; C, butyric acid; D, total SCFAs. $\bar{x}$ ± SEM (vertical bars) is presented for each group at different times: ○, YE (group treated with crude EPS); ▼, YB (group treated with *B. animalis* subsp. *lactis* INL1); ◆, YEB (group treated with both ingredients); ●, Y (control group). Different letters indicate significant differences among groups at a fixed

time point, while different number of asterisks indicates significant differences throughout time of treatment for each group separately. Concentrations at t_0 were subtracted for each mouse. Determinations were done in triplicate.

Fig. 4. Principal components analysis for different bacterial groups and organic acids determined at 25 days of treatment. A) score and B) loading graphs are presented: ○, YE (group treated with crude EPS); ▼, YB (group treated with *B. animalis* subsp. *lactis* INL1); ◆, YEB (group treated with both ingredients); ●, Y (control group).

Fig. 5. Boxplots for cytokines analysis in small intestine (A; asterisks indicate outliers), and bar plot for s-IgA levels in intestinal fluid (B; $\bar{x} \pm$ SEM is represented): Y, control group; YB, group treated with *B. animalis* subsp. *lactis*INL1; YE, group treated with crude EPS; YEB, group treated with both ingredients. Assays were done in triplicate.

Fig. 6. Histological analysis of the distal small intestine (jejunum and ileum) by May Grünwald Giemsa staining (10×). A, control group (Y); B, group treated with crude EPS (YE); C, group treated with both ingredients (YEB); D, group treated with *B. animalis* subsp. *lactis* INL1 (YB).

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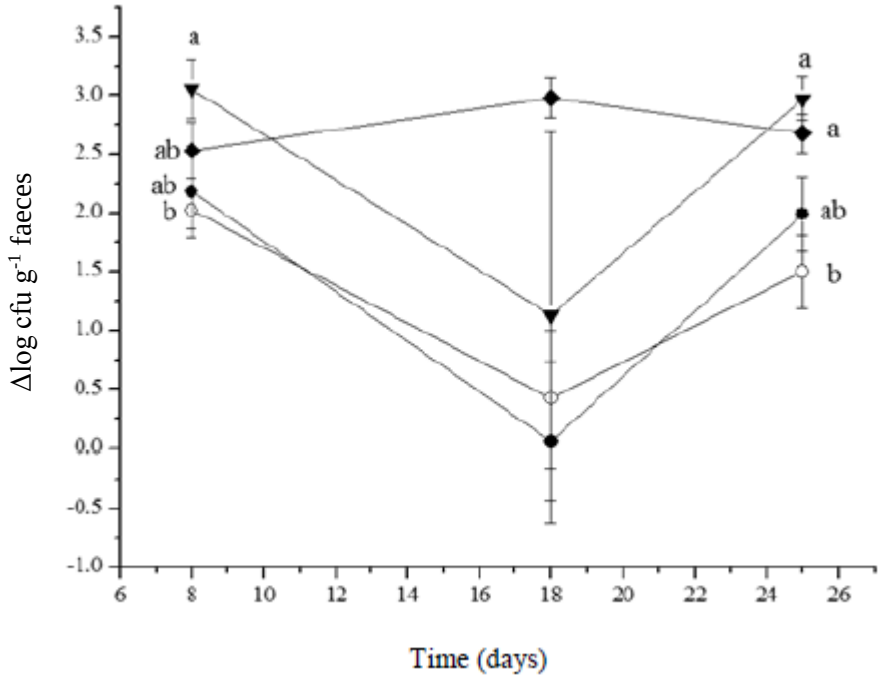
Table 1

Preliminary in vitro assay with crude (60 µg mL^{-1}) or purified (12.6 µg mL^{-1}) EPS extract with THP-1 cell line. [a]

Cytokines	Sample	$\bar{x}$ ± SD (pg mL^{-1})
TNF-α	Crude extract	3 ± 3^{b}
	Purified extract	433 ± 161^{a}
	Negative control	0.5 ± 0.1^{b}
	LPS	289 ± 45^{a}
IL-6	Crude extract	11 ± 2^{b}
	Purified extract	10 ± 1^{b}
	Negative control	8 ± 2^{b}
	LPS	23 ± 2^{a}
IL-10	Crude extract	37 ± 30^{a}
	Purified extract	28 ± 10^{a}
	Negative control	N.D.
	LPS	N.D.

[a] Different superscript letters indicate significant differences between groups for each cytokine analysed ($p < 0.05$). LPS, positive control; N.D., not detected.

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Figure 1

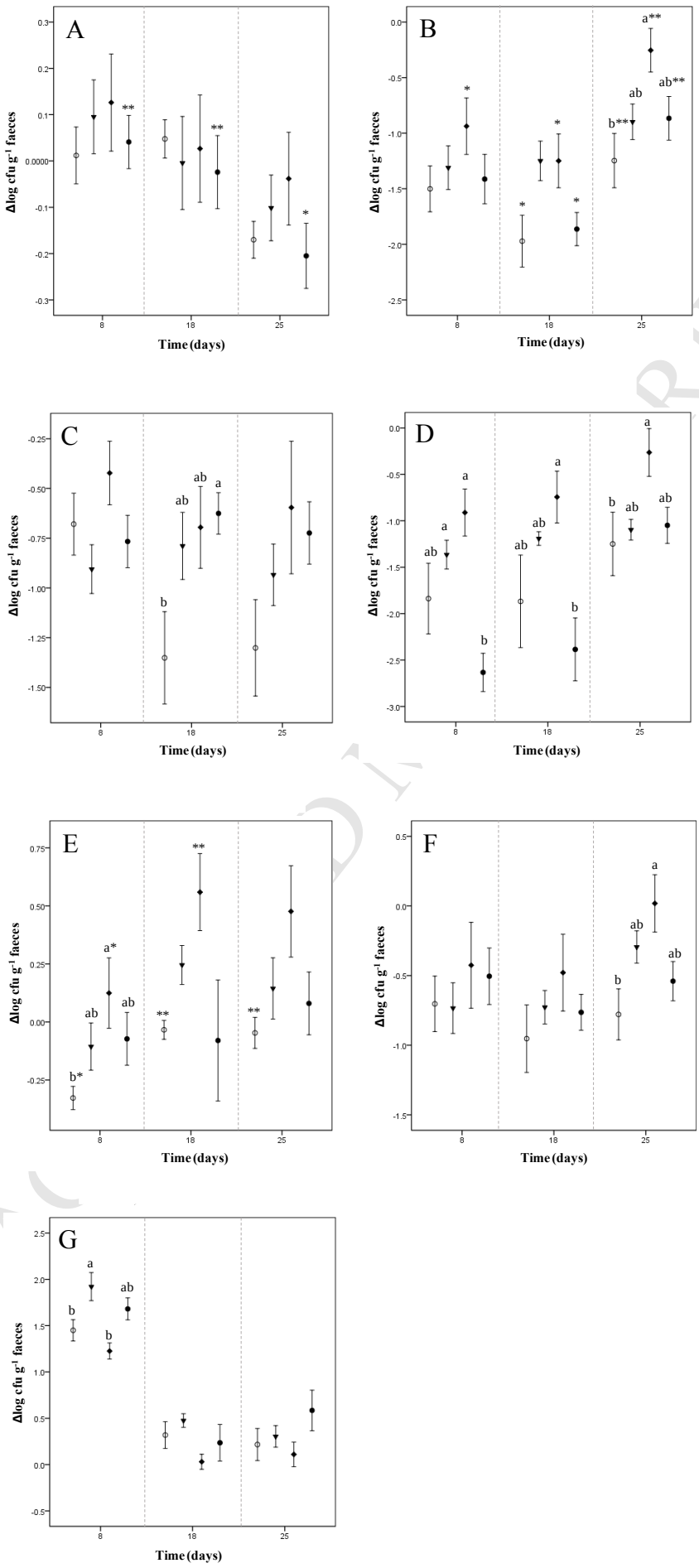


Figure 2

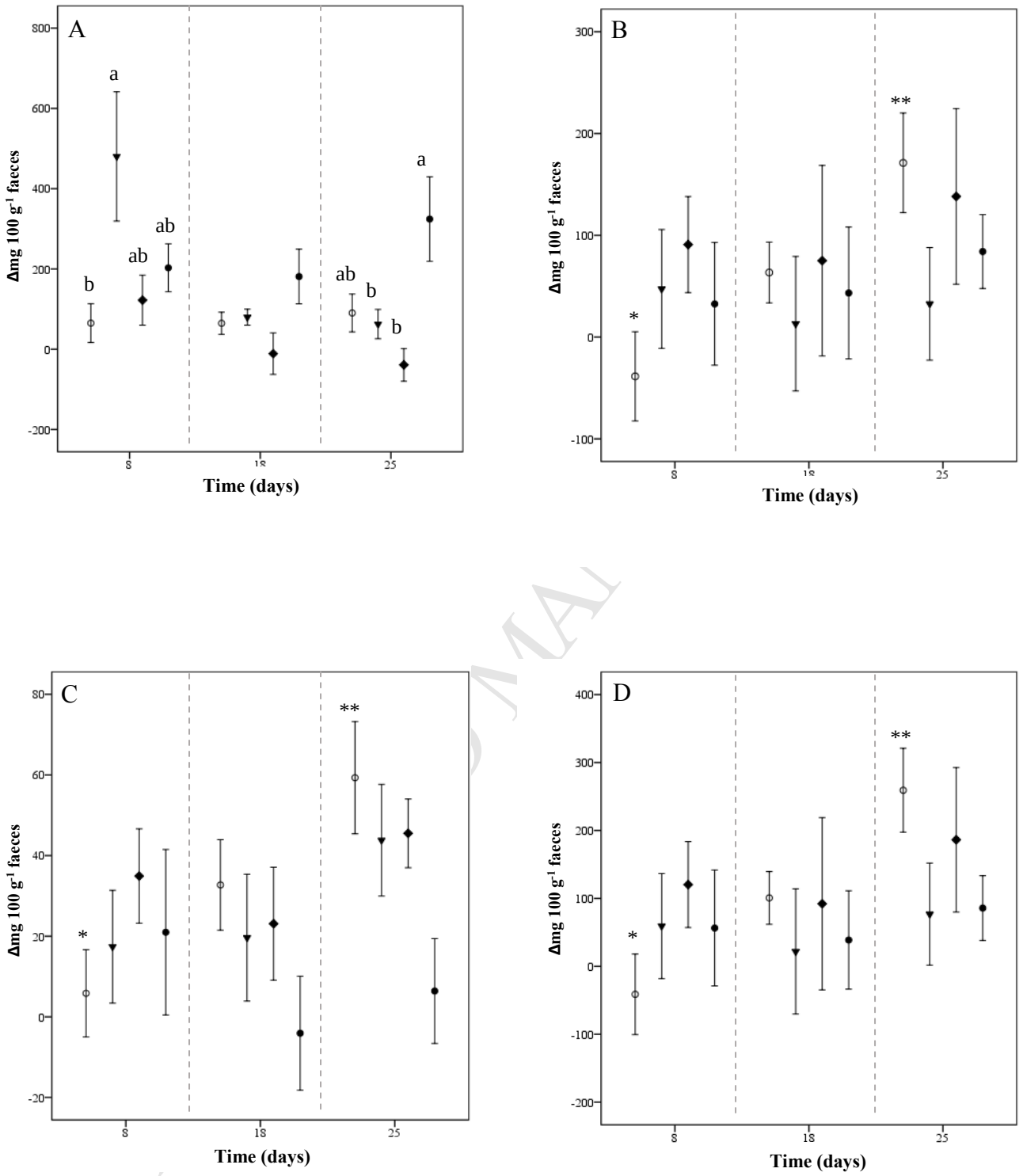
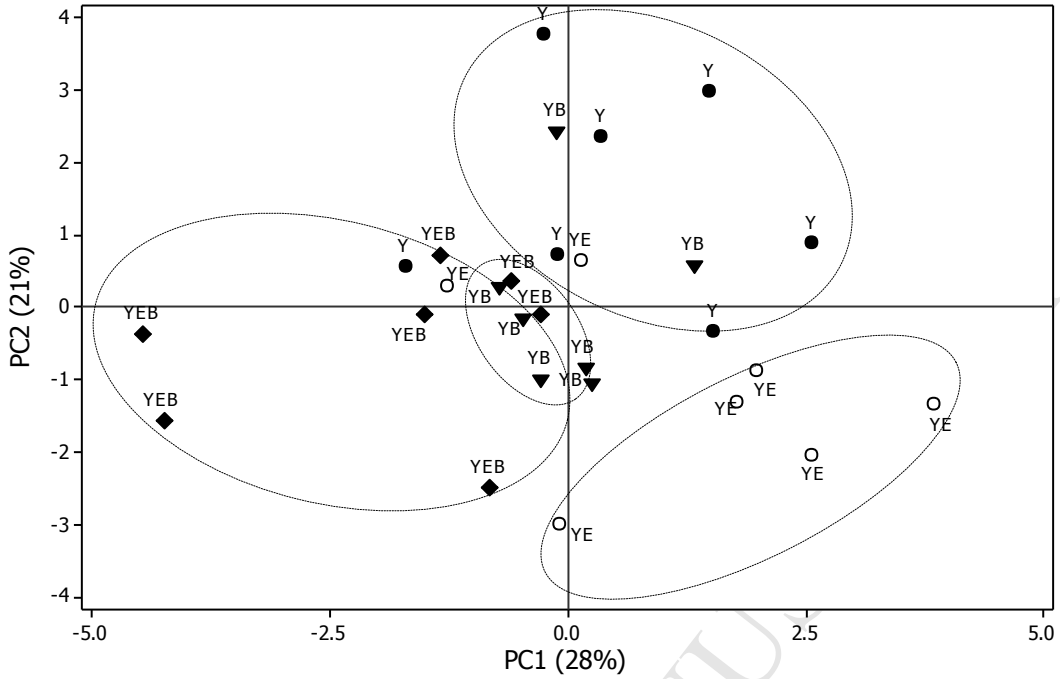


Figure 3

A)


PC2 (21%)
PC1 (28%)
Y
YB
YE
YEB

B)

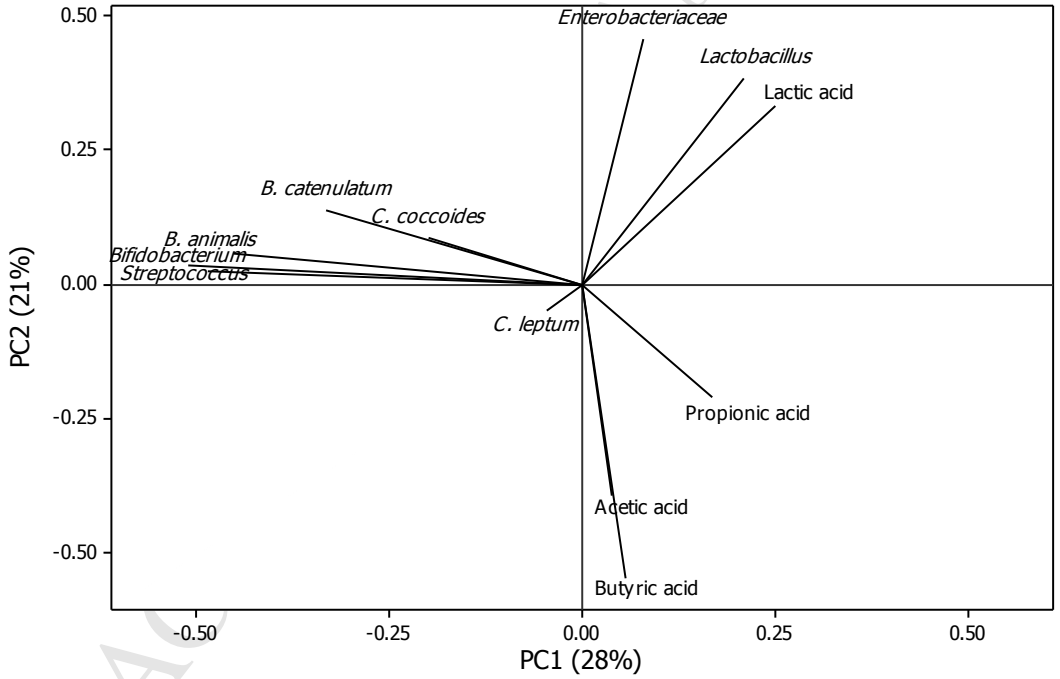
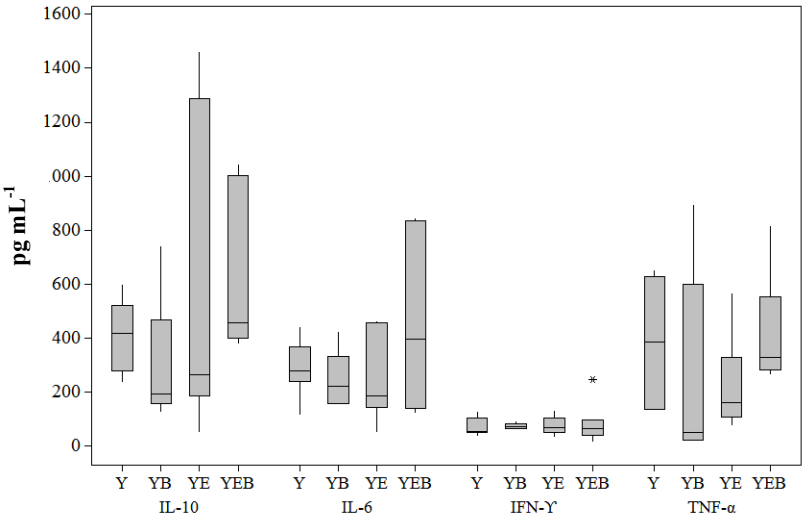
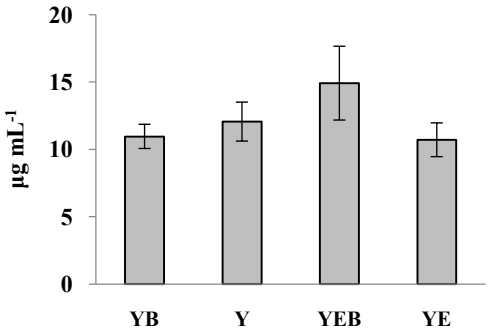

Enterobacteriaceae
Lactobacillus
Lactic acid
B. catenulatum
C. coccoides
B. animalis
Bifidobacterium
Streptococcus
C. leptum
Propionic acid
Acetic acid
Butyric acid
PC2 (21%)
PC1 (28%)

Figure 4

A)

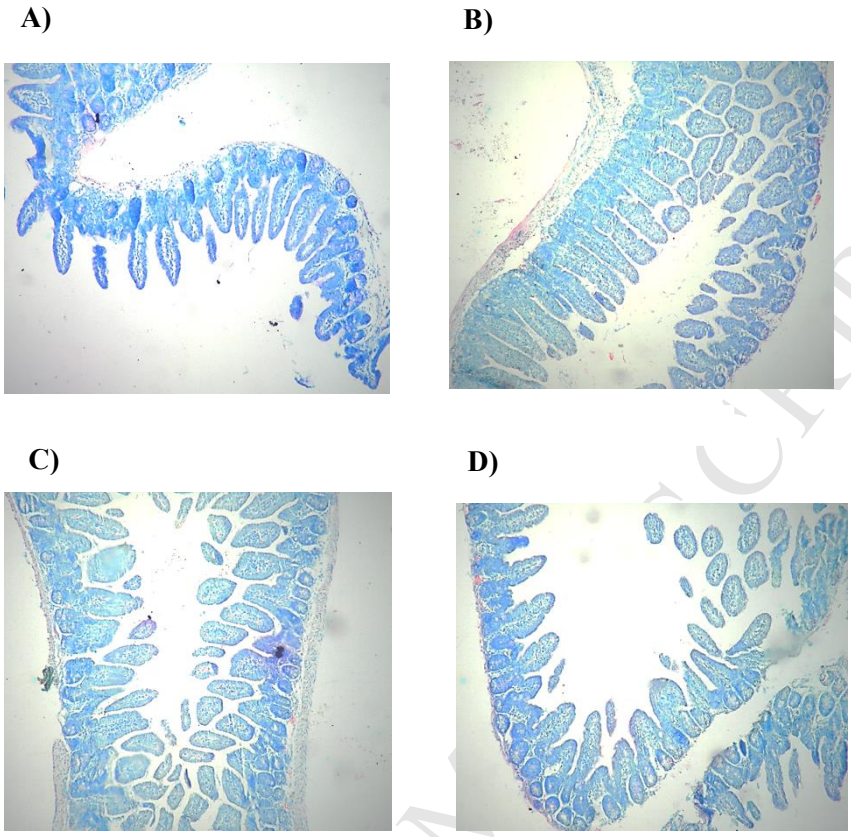


B)



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Figure 5



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Figure 6