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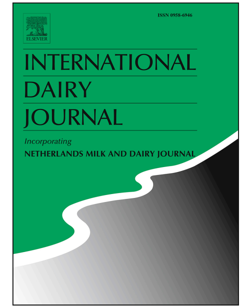

Functional properties of exopolysaccharide (EPS) extract from *Lactobacillus fermentum* Lf2 and its impact when combined with *Bifidobacterium animalis* INL1 in yoghurt

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PII: S0958-6946(19)30115-3

DOI: https://doi.org/10.1016/j.idairyj.2019.04.014

Reference: INDA 4500

To appear in: *International Dairy Journal*

Received Date: 5 July 2018

Revised Date: 17 April 2019

Accepted Date: 28 April 2019

Please cite this article as: Ale, E.C., Jean-Baptiste Bourin, M., Peralta, G.H., Burns, P.G., Ávila, O.B., Contini, L., JorgeReinheimer, Binetti, A.G., Functional properties of exopolysaccharide (EPS) extract from *Lactobacillus fermentum* Lf2 and its impact when combined with *Bifidobacterium animalis* INL1 in yoghurt, *International Dairy Journal*, https://doi.org/10.1016/j.idairyj.2019.04.014.

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

27

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

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42 **1. Introduction**

43

44 Whereas probiotics are live microorganisms that when administered in adequate
45 amounts confer a health benefit on the host (Hill et al., 2014), prebiotics are non-viable
46 substrates that function as nutrients for beneficial microorganisms harboured by the host,
47 including administered probiotic strains and indigenous microorganisms. Thus, a prebiotic
48 should elicit a metabolism biased towards health-promoting microorganisms within the
49 indigenous ecosystem. According to the consensus statement by ISSAP, the definition of a
50 prebiotic has been recently modified to 'a substrate that is selectively utilized by host
51 microorganisms providing a health benefit' (Gibson et al., 2017), including the impact of
52 prebiotic at extraintestinal sites: on bone strength, neural and cognitive processes, immune
53 functioning, skin, and serum lipid profile (Collins & Reid, 2016; Gibson et al., 2017). In
54 addition, the term synbiotics refers to a combination of both, probiotics and prebiotics in a
55 synergic way, so this term should be reserved for products in which the prebiotic
56 compound(s) selectively favours the probiotic organism(s) (Cencic & Chingwaru, 2010).
57 The catabolism of prebiotic carbohydrates by the metabolic activity of the gut
58 microbiota primarily produces three short chain fatty acids (SCFAs): acetate, propionate
59 and butyrate. The most abundant SCFA in the colon is acetate and, in general, represents
60 more than half of SCFA content detected in faeces (Louis, Scott, Duncan, & Flint, 2007).
61 The prebiotic substrates are able to selectively promote the growth of beneficial
62 microorganisms and induce changes in the levels of these acids in healthy individuals
63 (Lecerf et al., 2012). Thus, the levels of these SCFAs represent an indirect measure of the
64 level of beneficial microorganisms and their impact on human health.

65 Some lactic acid bacteria (LAB) are able to produce exopolysaccharide (EPS) as
66 part of their metabolism; these polymers can be released to the medium exerting
67 technological and functional roles. Regarding their functional properties, EPS from LAB
68 have demonstrated several benefits including bifidogenic/prebiotic effects,
69 immunomodulatory properties, prevention of pathogenic bacteria, gastritis, antitumor and
70 antioxidant activities, among others (Ale et al., 2016a; Hamet, Medrano, Pérez, &
71 Abraham, 2016; Polak-Berecka, Waśko, Szwajgier, & Chomaz, 2013; Rodríguez, Medici,
72 Rodríguez, Mozzi, & Font de Valdez, 2009; Wang et al., 2014).
73 In this regard, Sarikaya, Aslim, and Yuksekdag (2017) reported that the lyophilised
74 EPS from *Lactobacillus fermentum* LB-69 presented both bifidogenic and anti-biofilm
75 effects (in vitro) against a strain of *Bacillus cereus.* Furthermore, *Lactobacillus rhamnosus*
76 E/N is a probiotic strain that synthesises EPS with significant bifidogenic and antioxidant
77 activities (Polak-Berecka et al., 2013). These positive aspects make EPS, as well as the
78 producer strains, suitable ingredients for the formulation of novel functional foods.
79 In general, EPS are produced in situ during fermentation process of dairy food
80 when, for example, EPS^+ starter or adjunct cultures are added to the food matrices to
81 improve the textural and organoleptic characteristics of the final product (Amatayakul,
82 Sherkat, & Shah, 2006; Hassan, Ipsen, Janzen, & Qvist, 2003). Only a few studies have
83 described the effects of the direct application of bacterial EPS extracts as food additives.
84 For example, a crude EPS from *Streptococcus thermophilus* suspended in milk was
85 proposed to effectively prevent or heal chronic gastritis in a murine model (Rodríguez et
86 al., 2009; Rodríguez, Medici, Mozzi, & de Valdez, 2010). So far, no report describes the
87 application of EPS extracts as food ingredients in yoghurt, this option being an interesting

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

90 *L. fermentum* Lf2 is an autochthonous strain that was isolated as non-starter culture
91 from a local semi-hard cheese with blowing defects. This strain produces high amounts of
92 EPS when it grows under controlled conditions of temperature (30 ºC) and pH (6.0),
93 reaching 0.8 g L^{-1}, approximately, in semi-defined medium (SDM; Kimmel & Roberts,
94 1998) broth (Ale et al., 2016b). The total EPS is composed mainly of two polysaccharides:
95 a β-glucan whose repeating unit is a trisaccharide (1.8×10^3 KDa), and a
96 heteropolysaccharide constituting a disaccharide repeating unit of glucose and galactose,
97 the main backbone being composed of α-(1→6) linked galactose residues, each one
98 substituted by a terminal glucose residue (90 KDa) (unpublished data). From previous
99 studies, this EPS extract presented not only interesting technological characteristics, such as
100 an increase in consistency and pseudoplasticity of yoghurts (Ale et al., 2016b), but also
101 positive health effects, protecting against a *Salmonella* infection and increasing the levels
102 of IgA in intestinal fluid of mice (Ale et al., 2016a).

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

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

122

123 **2. Materials and methods**

124

125 *2.1. Organisms and growth conditions*

126

127 *L. fermentum* Lf2 and *B. animalis* subsp. *lactis* INL1 (INLAIN collection) were
128 stored at –80 °C in MRS (De Man, Rogosa and Sharpe, Biokar, Beauvais, France) broth
129 plus 15% (v/v) glycerol. They were routinely grown in MRS broth at 37 °C for 16 h in
130 aerobiosis and anaerobiosis (Anaeropack Anaero, Mitsubishi Gas Chemical Co., Inc.,
131 Germany) plus 0.1% (w/v) cysteine, respectively.
132 For yoghurt production, two commercial strains, *Streptococcus thermophilus* SC42
133 and *L. delbrueckii* subsp. *bulgaricus* 254 (both from Biochemical, Argentina), were
134 selected based on their inability (visual test) to produce EPS in milk. They were routinely

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

137 To design the calibration curves for qPCR analysis (Supplementary material, Table
138 S1,), the following strains were used as well: *Bifidobacterium breve* 110 (INLAIN
139 collection) and *Bifidobacterium bifidum* ATCC 35914 were cultured in the same way as *B.*
140 *animalis* subsp. *lactis* INL1. *S. thermophilus* SC42 was grown in Elliker broth (Biokar) at
141 42 °C; *Escherichia coli* EC101 in LB (Luria-Bertani) broth, with continuous agitation, at 37
142 °C under aerobiosis and *Staphylococcus aureus* 76 (INLAIN collection) was developed in
143 tryptic soy (TS) broth (Biokar) at 37 °C in aerobiosis. All cultures were grown from an
144 isolated colony.

145

146 *2.2. EPS production of* L. fermentum *Lf2*

147

148 EPS production was carried out as described by Ale et al. (2016b). Briefly,
149 cultivations were performed in a 2-L fermenter (Sartorius Biostat A plus®, Goettingen,
150 Germany) in SDM (Kimmel & Roberts, 1998) broth with the aim to minimise interferences
151 in EPS isolation by replacing yeast extract, beef extract and proteose peptone from MRS
152 broth by yeast nitrogen base and Bacto Casitone (both from Difco, Becton, Dickinson and
153 Company, Le Pont de Claix, France). *L. fermentum* Lf2 was inoculated from an overnight
154 culture (0.1%, v/v) and incubations were made at 30 °C for 72 h, with agitation (6 rpm) and
155 sparging with CO_2 (0.2 L min^{-1}). The pH was kept automatically at 6.0 with sterile 8 M
156 NaOH. After incubation, bacteria were removed by centrifugation (19,630 × *g*, 30 min, 5
157 °C) and EPS was extracted and precipitated at 4 °C for 48 h by adding 2 volumes of chilled
158 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

183 lipopolysaccharide (LPS, 0.5 μg mL^{-1}) was also included. The cells were incubated at 37
184 °C, with 5% CO_2 for 4 h for the detection of TNF-α, and 8 h for IL-6 and IL-10
185 determinations. The cytokine analysis was performed from the culture supernatants using
186 the DuoSet ELISA kits (R&D Systems, Minneapolis, United States) according to the
187 protocols recommended by the supplier. A negative control with untreated cells was also
188 included. All determinations were made in quadruplicate.
189
190 *2.4. In vivo assay to analyse the functional role of EPS as food ingredient in yoghurt*
191
192 *2.4.1. Manufacture of yoghurts*
193 Yoghurts were made with 10% (w/v) RSM inoculated with *S. thermophilus* SC42
194 and *L. delbrueckii* subsp. *bulgaricus* 254 (10^6 and 10^5 cfu mL^{-1}, respectively), with 0
195 (control) and 600 (equivalent to 146 mg pure EPS L^{-1}) mg L^{-1} of crude EPS added. The
196 incubation was at 43 °C until a final pH value of 4.6 was reached. The concentration of
197 crude EPS was chosen according to a previous study in which the crude extract at 600 mg
198 L^{-1} exerted protection against *Salmonella* infection (Ale et al., 2016a). After fermentation,
199 yoghurts were immediately cooled and stored at 4 °C. The following day an overnight
200 culture of *B. animalis* subsp. *lactis* INL1 was washed twice with sterile PBS and
201 resuspended in yoghurt with 0 or 600 mg L^{-1} of EPS extract at a level of 5×10^8 cfu mL^{-1};
202 this way the final cell count was not reduced during yoghurt manufacture.
203
204 *2.4.2. Animals and feeding procedures*
205 For in vivo trials, 6-week old male BALB/c mice weighing 19–21 g were obtained
206 from the random-bred colony of the Centro de Experimentaciones Biológicas y Bioterio

207 (FCV-UNL, Esperanza, Santa Fe, Argentina). Animals were humanly handled according to
208 the National Institutes of Health guide for the care and use of Laboratory animals (NIH
209 8023, 1978). In addition, all the procedures were approved by the Ethical Committee for
210 Animal Experimentation (FCV-UNL, Esperanza, Santa Fe, Argentina), and were made
211 following the recommendations of the INLAIN animal facility (Zacarías et al., 2014).
212 Twenty eight mice were clustered in four groups (seven mice/group) and received,
213 during 25 consecutive days and by gavage, 300 µL d^{-1} of any of the following samples: Y,
214 yoghurt; YE, yoghurt + 600 mg EPS L^{-1} (equivalent to 9 mg kg^{-1} d^{-1}); YB, yoghurt + 5×10^{8}
215 cfu mL^{-1} *B. animalis* subsp. *lactis* INL1; YEB, yoghurt + 600 mg EPS L^{-1} + 5×10^{8} cfu mL^{-1}
216 *B. animalis* subsp. *lactis* INL1. All animals simultaneously and ad libitum received sterile
217 tap water and a conventional balanced diet (Cooperación, Buenos Aires, Argentina). The
218 diet composition was the following: 230 g kg^{-1} protein, 60 g kg^{-1} crude fibre, 100 g kg^{-1}
219 total minerals, 13 g kg^{-1} Ca, 8 g kg^{-1} de P, 120 g kg^{-1} water and vitamins.
220
221 *2.4.3. Bifidobacteria counts in faeces*
222 Faeces samples were obtained at 0, 8, 18 and 25 days by the use of individual
223 metabolic cages (Tecniplast, Buguggiate, Italy) for cell counts of total bifidobacteria, DNA
224 extraction and SCFA quantification. Total bifidobacteria were determined on RCM agar
225 (Reinforced Clostridial Medium, Biokar) with the selective supplement for bifidobacteria
226 MUP (mupirocin, Merck, Darmstadt, Germany) according to Fanning et al. (2012), at
227 initial time (before receiving treatment) and at 8, 18 and 25 days post-treatment. The
228 selection of this medium was based on the supplementation with MUP, an antimicrobial
229 compound that acts as a selective agent for the inhibition of anaerobic bacteria with the
230 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

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303 rehydrated in successive baths of toluene, ethyl alcohol and, finally, distilled water. The
304 coloration consisted in leaving the section 15 min in MG (37 °C), 40 min in Giemsa (37
305 °C) and two subsequent baths in distilled water. For the differentiation, the samples were
306 placed 30 s in acetic acid (dilution 1: 100) and 10 s in alcohol/acetone (50: 50). Finally, the
307 assembly was carried out, leaving the sections in toluene for a few seconds. A drop of
308 EUKITT® (Sigma Aldrich) was placed on each slide and they were allowed to dry at room
309 temperature. At least two sections were analysed per animal.

310

311 *2.5. Statistical analysis*

312

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

323

324 **3. Results**

325

326 *3.1. Preliminary in vitro assay to analyse the immunomodulatory role of EPS*

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327

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

340

341 *3.2. In vivo assay to analyse the functional role of EPS as a food ingredient in yoghurt*

342

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

347

348 *3.2.1. Bifidobacteria counts in faeces*

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

351 days of treatment, significant differences were found between YE and YB groups, the latter
352 presenting approximately one more order than the first. At 18 days no significant
353 differences were seen between groups, probably due to the high variability obtained, and, at
354 the end of the treatment. No group differed from the control group.
355 The reason for the great variability observed (mainly at 18 days of treatment)
356 remains unknown, but it could be due to intrinsic variability among mice population, as
357 well as to the methodology employed for bacterial count. Although cell morphology was
358 checked for all colonies, possibly some of them were not effectively bifidobacteria. DNA
359 sequencing of each colony would have been needed to confirm its nature, but it was beyond
360 the scope of this work.
361
362 *3.2.2. Estimation of the levels of different bacterial groups in faeces by qPCR*
363 The universal primers were designed to have specificity with the conserved regions
364 of the rRNA-16S of prokaryotic cells (Baker, Smith, & Cowan, 2003), so they are related to
365 the total microbial load present in the intestine. In this case, no differences were observed
366 between groups at each time evaluated, but a significant effect was observed for the control
367 group (Y) during the time of treatment (Fig. 2A). At 25 days the levels of total bacteria
368 were significantly lower ($p < 0.05$) than those observed at 8 and 18 days. For the remaining
369 groups there were no significant differences throughout time.
370 When the levels of the genus *Bifidobacterium* were estimated, significant
371 differences were detected for the factors time and treatment (Fig. 2B). Differences among
372 treatments were observed only at 25 days for the group YEB, which presented values
373 significantly higher than the group YE ($p < 0.05$), but none differed from the control group
374 (Y) and group YB. This was also confirmed by the cell count estimations, since at 25 days,

375 the group YEB presented higher levels of this genus in comparison with the group YE (Fig.
376 1). When evaluating the influence of time on each treatment, the control group (Y) and the
377 group that received only EPS (YE) presented levels significantly lower at 18 days than
378 those obtained at 25 days, indicating that the population of bifidobacteria increased towards
379 the end of the assay. No significant differences were observed during time for YB
380 treatment, while the levels obtained after 25 days were higher than those observed at 8 and
381 18 days for YEB group ($p < 0.05$). Thus, considering both factors, the results suggest that
382 the combination of the two ingredients, EPS and the bifidobacteria, would play a possible
383 synergic role, since the levels of the genus *Bifidobacterium* at the end of the treatment
384 remained higher than those obtained at 8 and 18 days. It should be highlighted that, for the
385 statistical analysis of the design applied, when the presence of repeated measures over time
386 is considered as another source of variability, a better estimation of the error is produced.

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

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

399 was appreciated (Fig. 2D). After 8 days, the groups that received the bifidobacteria (YB
400 and YEB) presented significantly higher levels than the control group (Y). Although this
401 increment could be associated to the administration of the probiotic strain, it is interesting
402 to evaluate the estimations after 18 days. In this case, the only group that presented
403 significant differences in comparison with the control group was YEB, indicating that the
404 combination of EPS and bifidobacteria was more effective in increasing the levels of the
405 species *B. animalis* at this time. At 25 days, only significant differences were observed ($p <$
406 0.05) between groups YEB and YE, but none was different from the control group.

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

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

421

422 *3.2.3. Quantification of SCFAs in faeces*

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423 Fig. 3A shows the results obtained for lactic acid; although it is not considered a
424 SCFA, its levels are related to the metabolism of LAB and other intestinal bacteria. Also, it
425 is feasible to be converted to butyrate and propionate by the gut microbiota (Bourriaud et
426 al., 2005). In our study, after 8 days, group YB showed significantly higher concentrations
427 of lactic acid than YE, but none was different from the control group. At 18 days no
428 differences were observed between treatments, while at 25 days the control group Y
429 presented higher lactic acid levels ($p < 0.05$) than the groups that received the
430 bifidobacteria, either alone or together with the EPS. These results suggest that, despite the
431 fact that the group YB reached high levels of lactic acid at 8 days, these levels were
432 significantly lower at the end of treatment in comparison with the control group, either in
433 combination or not with the EPS of *L. fermentum* Lf2.
434 Regarding acetic acid (Fig. 3B), the group YE presented levels significantly higher
435 at the end of the treatment in comparison with 8 days. For the other treatments, no
436 significant changes were observed during the 25 days of intervention, or among the four
437 groups at each time analysed. The same observations can be made for the results obtained
438 for butyric acid and the sum of the three SCFAs (Fig. 3C and 3D, respectively), showing an
439 increment in the levels of both acids for group YE. For propionic acid, despite presenting a
440 similar behavior, no significant differences were observed among groups or during
441 treatment (data not shown).
442 These results indicate that the treatment that favours the production of acetic acid,
443 butyric acid and the sum of the three SCFAs is YE. In the case of the combination of both
444 ingredients (group YEB), no significant differences were observed during treatment.
445 Considering these results, it seems that EPS alone is able to exert a clear effect on the
446 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

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471 extract (YE and YEB) presented higher values of the regulatory cytokine IL-10 than the
472 other groups, no significant differences were observed for all the cytokines evaluated. Fig.
473 5B shows s-IgA concentrations in intestinal fluid and no significant differences were
474 observed between treatments, probably because the maximum peak occurred before 25
475 days. This could be justified considering that a significant increase in s-IgA concentration
476 was observed at 15 days of treatment, when the EPS was added to yoghurt at half the
477 concentration used in the present assay (Ale et al., 2016a).
478
479 *3.2.5. Histological analysis*
480 The applied treatments caused no damage to the intestinal mucosa since normal
481 morphology for both the small (Fig. 6) and large intestines (data not shown) of all the mice
482 was observed. None of the tissues exhibited signs of inflammation or lymphocytic
483 infiltration towards the mucosa and submucosa.
484
485 **4. Discussion**
486
487 By in vitro analysis, we could demonstrate that EPS from *L. fermentum* Lf2, in its
488 purified form, caused an increase in the levels of the proinflammatory cytokine TNF-α,
489 while both forms of EPS (purified and crude) produced an increase in the regulatory
490 cytokine IL-10. As TNF-α is an important proinflammatory cytokine, a stimulation of its
491 levels represents a typical immune tolerant phenotype. Both TNF-α and IL-6 play an
492 important role in the signalling system for the initiation of the mucosal inflammatory
493 response when the host intestinal epithelial surface is invaded by microbial pathogens (Jung
494 et al., 1995). In addition, IL-10 is essential for maintaining the integrity and homeostasis of

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

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

513 Considering the chemical and structural characteristics of the *L. fermentum* Lf2 EPS
514 extract (two uncharged fractions of low and high average molecular mass, unpublished
515 data), it seems that the low molecular mass polysaccharide could have directed the response
516 obtained, since it is present in a higher proportion than the high molecular mass
517 polysaccharide (unpublished data). According to our results, the purified EPS from *L.*
518 *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

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

548 When the levels of the genus *Bifidobacterium* were estimated by cell counts,
549 although it is more difficult to observe a clear tendency due to the variability of the results
550 among animals, at day 25 the difference detected seems to obey exclusively to the presence
551 of the bifidobacterium strain added. In this case, it would be more appropriate to considerer
552 the results of qPCR determination for this bacterial genus due to the high specificity of the
553 method. The qPCR results indicated that the combination of EPS and the probiotic strain
554 (group YEB) seems to be effective in increasing the levels of the total *Bifidobacterium*
555 during all the evaluated period (difference of 0.7 log cfu g^{-1}), approximately, between 8 and
556 25 days of treatment) when compared with the group of animals that received exclusively
557 *B. animalis* INL1 (group YB; difference of 0.4 log cfu g^{-1}), indicating that the combination
558 of both ingredients exerted a possible bifidogenic role. Salazar, Gueimonde, Hernández-
559 Barranco, Ruas-Madiedo, and de Los Reyes-Gavilán (2008) observed, by batch cultures of
560 faecal samples, that 11 different bifidobacterial EPS had a bifidogenic effect, detecting
561 (PCR-DGGE) changes of other microbial groups during fermentation, mainly of
562 *Bacteroides*, *E. coli* and microorganisms related to the second. Hamet et al. (2016) showed
563 by DGGE that, when kefiran was orally administered to BALB/c mice, it modified the
564 intestinal and faecal microbiota, increasing the population of bifidobacteria. Sarikaya et al.
565 (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
640 **Acknowledgements**
641
642 This work was financed by the Agencia Nacional de Promoción Científica y
643 Tecnológica (ANPCyT) and by the Consejo de Investigaciones Científicas y Tecnológicas
644 (CONICET) through the projects PICT-2014-2046 and PIP 2013 (112 201201 00124CO),
645 respectively. The in vitro assay was done through a collaboration with Prof. Paul O'Toole
646 (University College Cork) and his team, by means of a scholarship provided by the
647 Erasmus Mundus programme (AMIDILA).
648
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1 **Figure legends**

2

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

9

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

19

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

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25 time point, while different number of asterisks indicates significant differences throughout
26 time of treatment for each group separately. Concentrations at t_0 were subtracted for each
27 mouse. Determinations were done in triplicate.
28
29 **Fig. 4.** Principal components analysis for different bacterial groups and organic acids
30 determined at 25 days of treatment. A) score and B) loading graphs are presented: ○, YE
31 (group treated with crude EPS); ▼, YB (group treated with *B. animalis* subsp. *lactis* INL1);
32 ◆, YEB (group treated with both ingredients); ●, Y (control group).
33
34 **Fig. 5.** Boxplots for cytokines analysis in small intestine (A; asterisks indicate outliers), and
35 bar plot for s-IgA levels in intestinal fluid (B; $\bar{x}$ ± SEM is represented): Y, control group;
36 YB, group treated with *B. animalis* subsp. *lactis*INL1; YE, group treated with crude EPS;
37 YEB, group treated with both ingredients. Assays were done in triplicate.
38
39 **Fig. 6.** Histological analysis of the distal small intestine (jejunum and ileum) by May
40 Grünwald Giemsa staining (10×). A, control group (Y); B, group treated with crude EPS
41 (YE); C, group treated with both ingredients (YEB); D, group treated with *B. animalis*
42 subsp. *lactis* INL1 (YB).
43
44

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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	x̄ ± 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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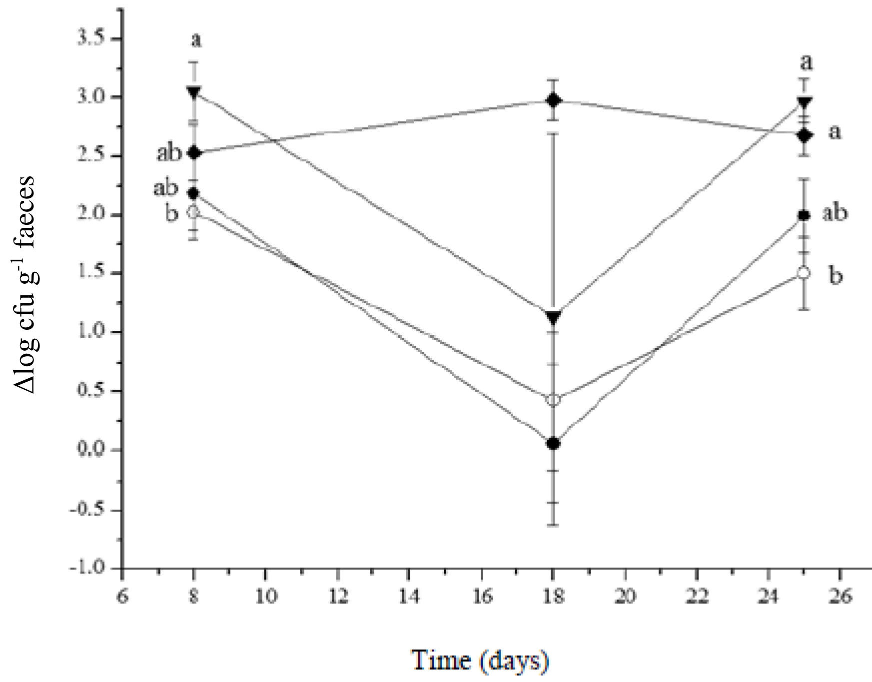

Figure 1

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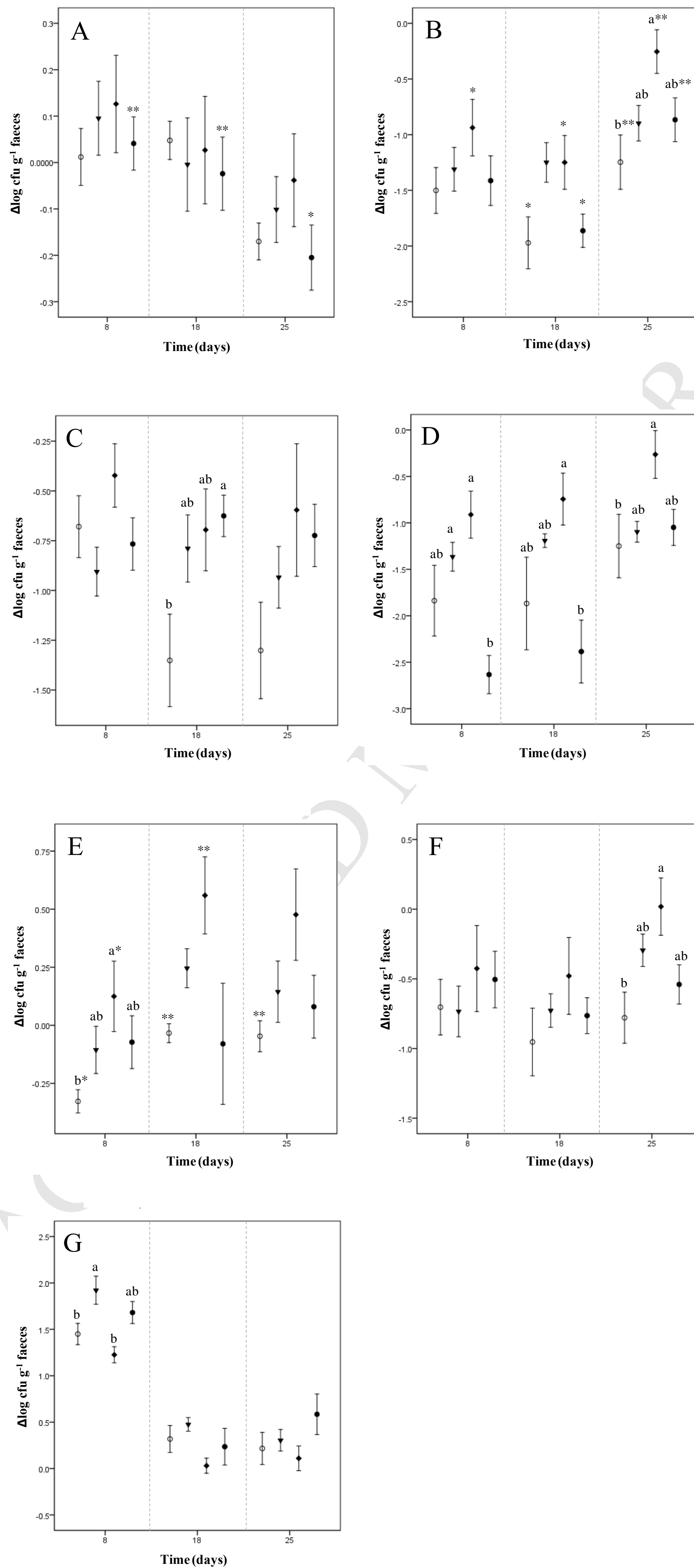

Figure 2

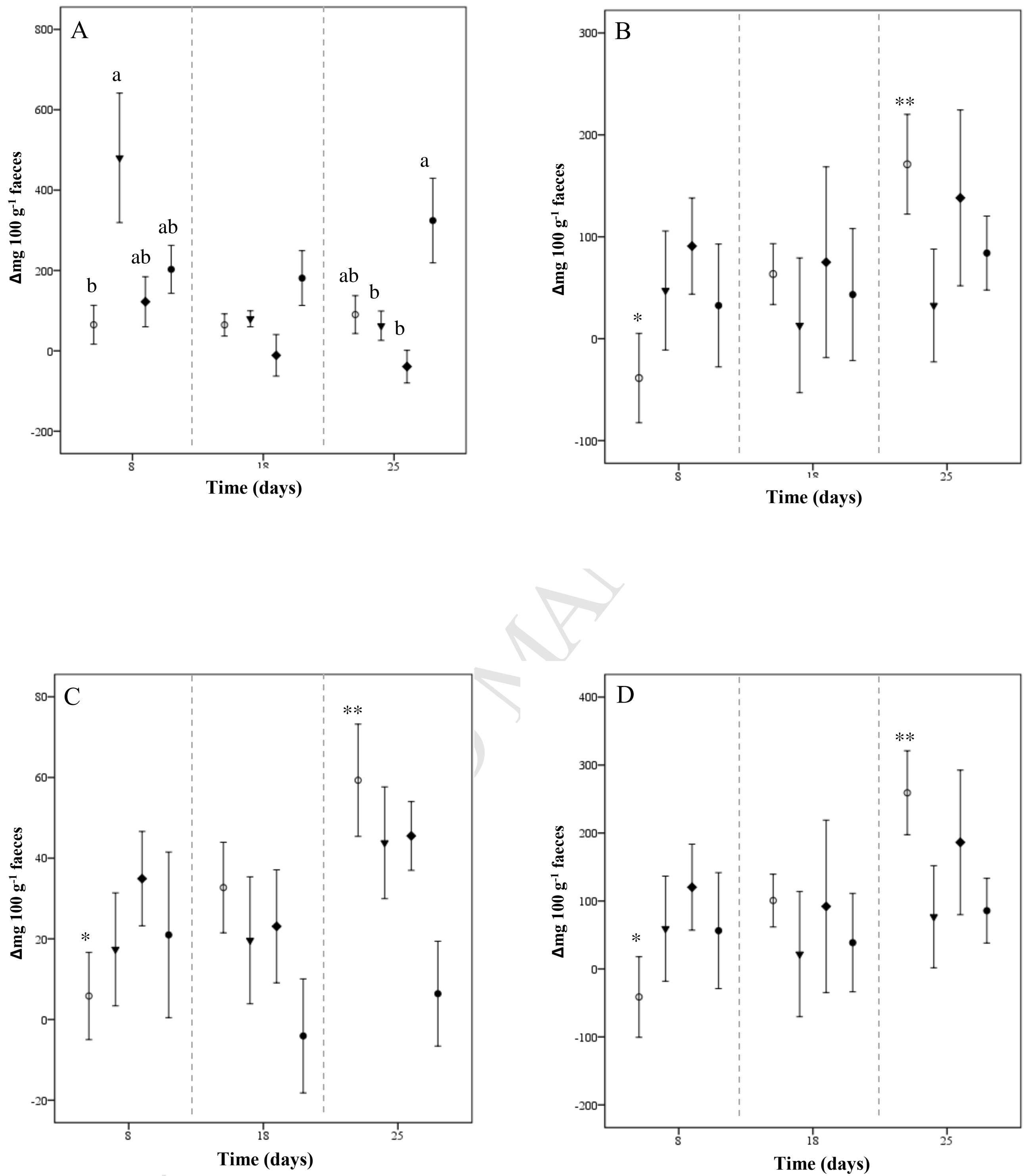

Figure 3

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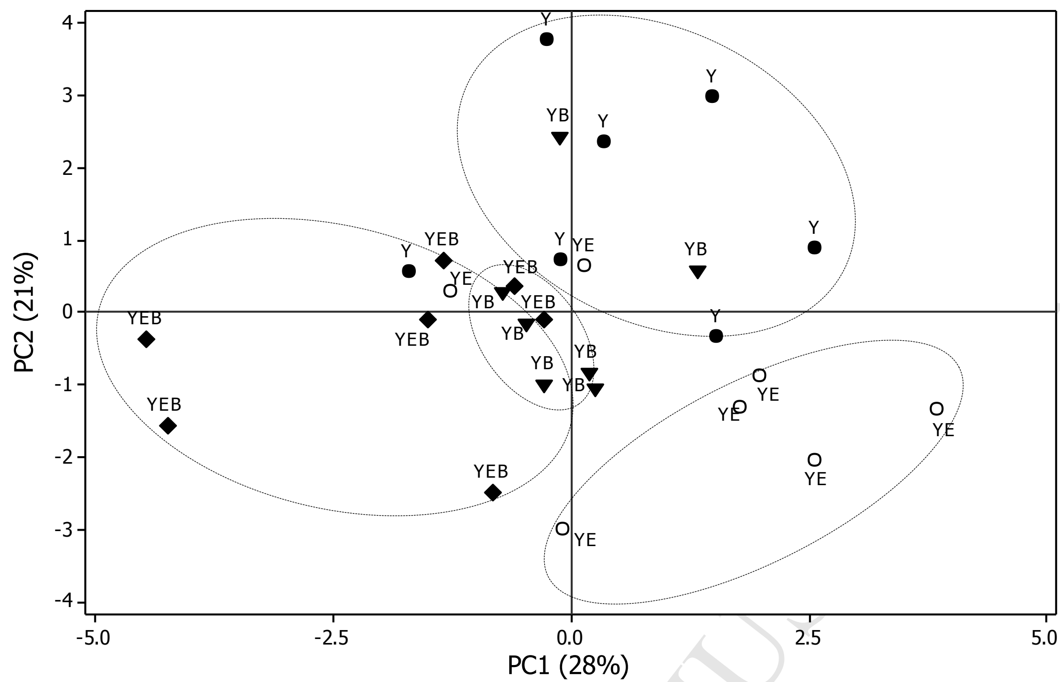
A)



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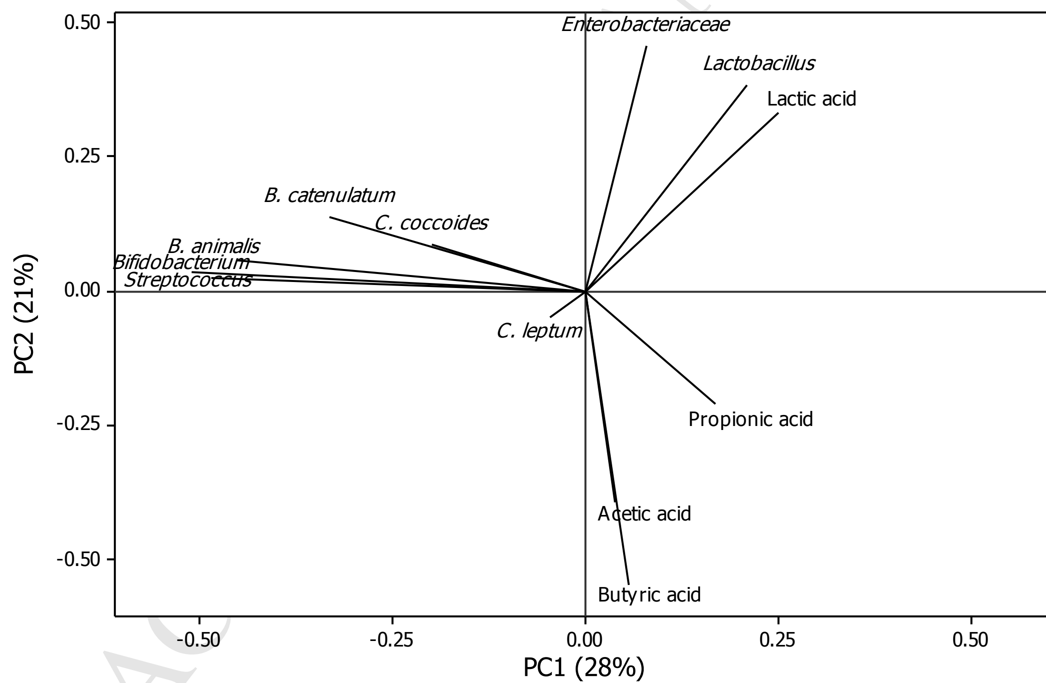
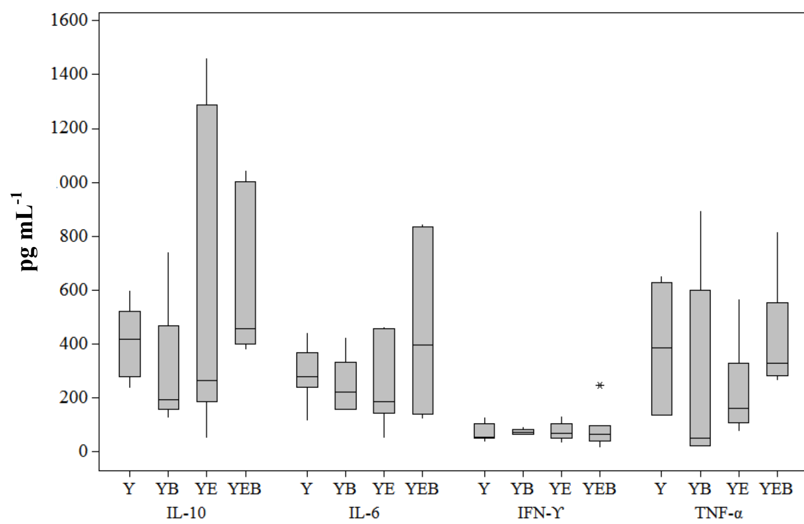


Figure 4

A)



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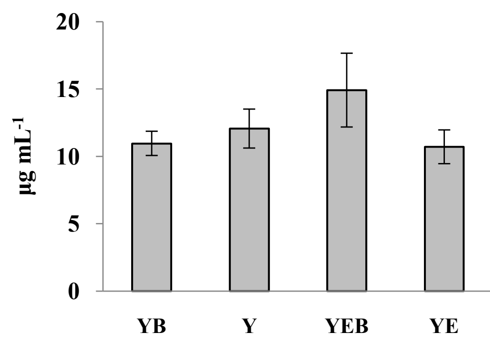

Figure 5

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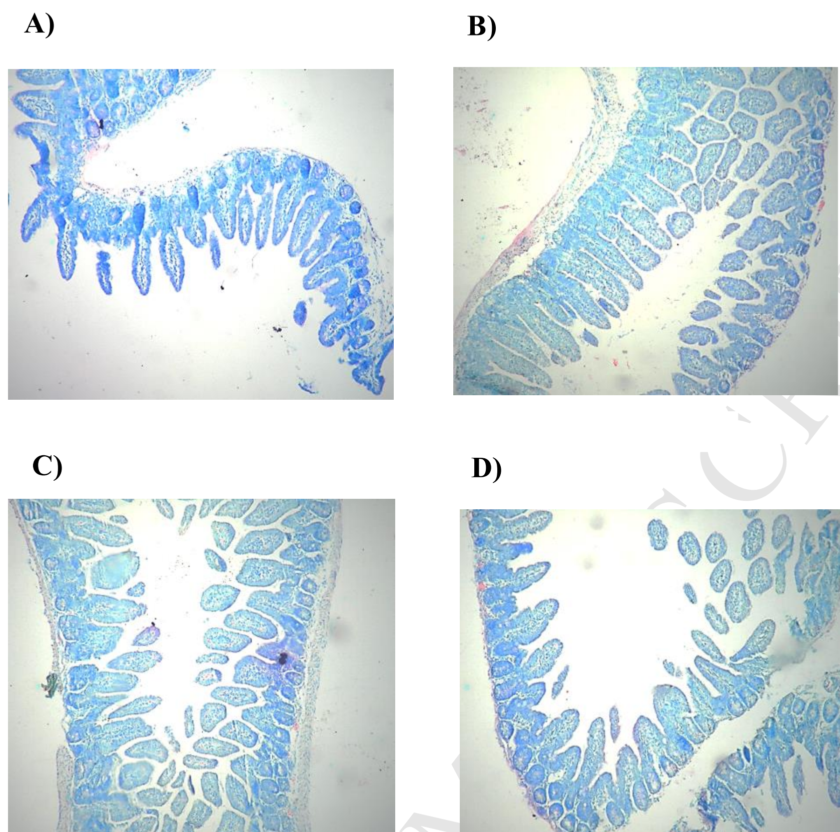

Figure 6

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