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Title: Safety and intestinal microbiota modulation by the exopolysaccharide-producing strains *Bifidobacterium animalis* IPLA R1 and *Bifidobacterium longum* IPLA E44 orally administered to Wistar rats.

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Short title:

Bifidobacterium animalis and *Bifidobacterium longum* administered to Wistar rats

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Bifidobacterium, Wistar rats, intestinal microbiota, modulation, exopolysaccharide

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ABSTRACT

Bifidobacterium animalis subsp. *lactis* IPLA R1 and *Bifidobacterium longum* IPLA E44 strains were tested *in vivo* for their safety and ability to modulate the intestinal microbiota. Chemically simulated gastrointestinal digestion showed considerably lower survival of E44 than R1 strain, the first microorganism also being more sensitive to refrigerated storage in 10%- skimmed milk at 4ºC. Harmful glycosidic activities were absent, or at low levels, in the strains R1 and E44. Both strains were sensitive to most antibiotics and resistant to aminoglycosides, a common feature in bifidobacteria. Similarly to several other bifidobacteria strains, *B. animalis* subsp. *lactis* IPLA R1 displayed a moderate resistance against tetracycline which correlated with the presence of *tet*(W) gene in its genome. The general parameters indicating well-being status, as well as translocation to different organs and histological examination of the gut tissues, revealed no changes induced by the administration of bifidobacteria. 12 week-old male Wistar rats were distributed into three groups, eight rats in each. Two groups were administered daily over 10^8 cfu of the corresponding strain suspended in 10%- skimmed milk for 24 days, whereas rats in the placebo group received skimmed milk without microorganisms added. The microbiota and short chain fatty acids (SCFA) were monitored in faeces at different time points during treatment and in caecum-content at the end of the assay. Quantitative PCR (qPCR) showed that faecal and caecal *Bifidobacterium* levels were higher in bifidobacteria-fed rats than in the placebo rats at the end of the intervention, whereas total anaerobic-plate counts did not show significant differences. Quantification of *B. animalis* and *B. longum* by qPCR showed that, independent of the microorganism administered, treatment with bifidobacteria resulted in higher levels of *B. animalis* in the caecum. PCR-DGGE analysis of microbial populations revealed a higher diversity of bands in caecum-content of rats fed *B. animalis* IPLA R1 than in the placebo group and rats fed *B. longum* IPLA E44. Remarkably, although no variations in

the proportion of acetate, propionate and butyrate were found, at the end of the assay the total SCFA concentration in the faeces of rats fed bifidobacteria was significantly higher and those in caecum-content significantly lower, than that of the placebo group. This suggests a displacement of the SCFA production to parts of the colon beyond the caecum in rats receiving bifidobacteria. Therefore, the oral administration of *B. animalis* IPLA R1 and *B. longum* E44 can be considered safe, these microorganisms having the ability to modulate the intestinal microbiota of rats by influencing SCFA and the bifidobacterial population levels.

INTRODUCTION

The distal gastrointestinal tract (GIT) works as an anaerobic bioreactor, composed of relatively few microbial phyla with high diversity at species/strain level, and it mediates important host physiological functions as well as chemical transformations of indigestible components of the diet (Bäckhed et al., 2005). This symbiotic relationship between intestinal microbiota and host is crucial for maintaining a health status and several intervention strategies have been developed to keep this homeostasis. The FAO/WHO defined probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host". A FAO /WHO expert consultation group has proposed a guideline in order to assess the health properties and safety considerations of probiotics intended for human food applications. Several rational criteria, based on *in vitro* and *in vivo* evidences, are currently recommended for the selection of putative probiotic strains before assessing their efficacy in human intervention studies (FAO/WHO 2006).

Lactobacillus and *Bifidobacterium* are the most frequently used probiotics for human consumption, and due to their long history of safe use, some species have the "Qualified Presumption of Safety" (QPS) status (EFSA, 2007). In recent years, our research group has been working with bifidobacteria strains isolated from human intestinal microbiota which are able to produce exopolysaccharides (EPS) (Ruas-Madiedo et al., 2007). Bacterial EPS have been claimed to play an important role in the putative probiotic effect of some producing strains (Ruas-Madiedo et al., 2008). The EPS synthesised by bifidobacteria are able to modify the adhesion of probiotics and entero-pathogens to human mucus (Ruas-Madiedo et al., 2006). It has also been suggested that bacterial EPS could play a protective role for the producing strain under adverse environmental conditions (Ruas-Madiedo et al., 2008). In addition, EPS from bifidobacteria can be used as fermentable substrates by the human intestinal microbiota (Salazar et al., 2008). Namely, we have recently shown that the EPS

produced by the strains *Bifidobacterium animalis* subsp. *lactis* IPLA R1 and *Bifidobacterium longum* subsp. *longum* IPLA E44 were able to modify levels of microbial intestinal populations and to promote shifts in the production of short chain fatty acids (SCFA) when tested in a pH-controlled human faecal model that simulates the distal part of the gut (Salazar et al., 2009). A suitable approach to modulate the intestinal microbiota, and thus to exert a health benefit, could be the use of the EPS-producing bifidobacteria. However, before embarking on long and expensive human intervention studies it is important to have good *in vivo* evidence, as well as to ascertain the safety of such strains. In this way, the aim of the present study was to determine the safety of strains *B. animalis* subsp. *lactis* IPLA R1 and *B. longum* subsp. *longum* IPLA E44 by means of several *in vitro* and *in vivo* tests, to ascertain their ability to survive the upper GIT challenge and to assess the capability of these strains to modulate the intestinal microbiota in an *in vivo* animal model.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Two EPS-producing strains were used in this study: *B. animalis* subsp. *lactis* IPLA R1 and *B. longum* subsp. *longum* IPLA E44, both held at the IPLA culture collection [GenBank accession numbers of their partial 16S rRNA gene sequence: EU430035 (Salazar et al., 2008) and GU586289 (this article), respectively]. Strains from frozen stocks were re-activated overnight at 37ºC in MRSC [MRS broth (Biokar Diagnostic, Beauvais, France) supplemented with 0.25% (w/v) L-cysteine (Sigma Chemical Co., St. Louis, MO, USA)] in an anaerobic chamber Mac500 (Don Whitley Scientific, West Yorkshire, UK) under 80% N_2 (v/v), 10% (v/v) CO_2 and 10% (v/v) H_2 atmosphere.

Suspensions of each strain were separately prepared in milk to be administered to experimental animals. Cultures grown overnight were used to inoculate (2% w/v) fresh

MRSC broth which was incubated for 24 h under the conditions previously indicated. Afterwards, cultures were washed twice with sterile PBS solution (8.0 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4 and 0.2 g/L KH_2PO_4; pH 7.3) and resuspended in sterile 10%-reconstituted skimmed milk (Difco™, Becton Dickinson, MD, USA) at a concentration of about $1x10^{10}$ cfu/mL. Two milk-bacterial suspension batches for each strain were prepared weekly and stored during a maximum of 4 days at 4ºC until administration to animals. To test the viability of the strains in the milk-bacterial suspensions under cold storage, serial dilutions in Ringer's solution (Merck, Darmstadt, Germany) were made and deep plated on agar-MRSC. Plates were incubated under anaerobic conditions for 72 h to determine the bifidobacteria counts (cfu/mL). The identity of the strains was tested in some batches by partially sequencing the 16S rRNA gene using Y1-Y2 primers (Salazar et al., 2009).

Simulated gastrointestinal transit of bifidobacteria

The survival of the two bifidobacteria strains to the GIT transit was studied in an *in vitro* model that chemically simulates physiological conditions, which had been modified from those previously described (Fernández et al., 2003). The following preparations were used: (i) gastric juice (GJ) containing 125 mmol/L NaCl, 7 mmol/L KCl, 45 mmol/L $NaHCO_3$, and 3 g/L pepsin (Sigma), pH 2.0 adjusted with HCl, (ii) duodenal juice (DJ) containing bovine 1% (w/v) bile (Sigma) pH 8.0 adjusted with 10 N NaOH, and (iii) intestinal juice (IJ) containing 0.3% (w/v) bovine bile, 0.1% (w/v) pancreatin (Sigma), pH 8.0 adjusted with 10 N NaOH. To simulate the GIT transit, the bacterial suspensions were sequentially submitted to the GJ, DJ and IJ conditions as follows. Cells from 24 h MRSC-grown cultures were harvested by centrifugation (10,000 *g*, 15 min, 5ºC), washed twice with 0.85% (w/v) NaCl and concentrated 10 fold. For each strain, 100 μL of the concentrated suspensions were centrifuged and resuspended either in 1 mL of GJ or in 1 mL of GJ

containing 10% skimmed milk, which increased the pH of the bacterial suspension to about 4.0. Bacterial suspensions were then incubated for 90 min at 37ºC with mild stirring (200 rpm). Afterwards, cells were harvested (10,000 *g*, 15 min), resuspended in DJ and incubated anaerobically for 10 min at 37ºC. After this step, cells were harvested again, resuspended in the IJ and incubated for 120 min at 37ºC in anaerobic conditions. Initially, and after each step, samples were taken to determine bacterial counts (cfu/mL) as previously indicated. Additionally, at the end of the simulation of GIT transit bacterial suspensions were collected and dyed with the Live/Dead® BacLight bacterial viability kit (Molecular Probes, Invitrogen, Merck) following the manufacturer's instructions. Fluorescence was measured in a Cary Eclipse fluorescence spectrophotometer (Varian Ibérica S.A., Madrid, Spain). The ratio between live (cultivable and non-cultivable, green colour) and dead (red colour) bacteria was used to calculate the percentage of survival.

Enzymatic activities and antibiotic resistance of bifidobacteria

Several enzymatic activities were determined using the semi-quantitative method Api-ZYM (BioMérieux, Montalieu-Vercieu, France) following the manufacturer´s instructions. The minimum inhibitory concentration (MIC) of eight antibiotics (gentamicin, kanamycin, streptomycin, neomycin, tetracycline, erythromycin, clindamycin and chloramphenicol) was determined against the strains, previously grown under conditions described by Klare et al. (2005), using the VetMic^{TM} Lact-I microdilution test (SVA, Uppsala, Sweden) following the manufacturer´s specifications. High antibiotic resistance levels were further corroborated by E-test strips (AB Biodisk, Solna, Sweden), as indicated by Ammor et al. (2008). Finally, the presence of tetracycline resistance genes encoding ribosomal protection proteins was determined by PCR using two pairs of degenerated primers, DI-DII and Tet1-Tet2, and

primers TetWF-Tet2 specific for the gene *tet*(W) according to the procedure described by Flórez et al. (2006).

Animal model and experimental design

The study was approved by the Animal Experimentation Ethical Committee of the Oviedo University, and subsequent handling strictly followed the European Communities Council Directive of November 24, 1986 (86/609/EEC). 12 week-old male Wistar rats (300-350 g body weight) were obtained from the Oviedo University Bioterio Facility. Animals were fed *ad libitum* a commercial diet (PanlabAO4, Panlab S.L., Barcelona, Spain) and were kept at 23-25ºC and 12/12 h light-dark cycle, each rat housed in an individual cage. Animals were divided into three groups (n = 8 rats per group): i) placebo group, ii) B1 group (fed *B. animalis* subsp. *lactis* IPLA R1) and iii) B2 group (fed *B. longum* subsp. *longum* IPLA E44).. The placebo group received 100 μL of sterile skimmed milk daily, and each rat of the B1 and B2 groups received a dose of 10^9 cfu per day of the corresponding milk-bifidobacteria suspension in a volume of 100 μL. Placebo and milk-bifidobacteria suspensions were orally administered for 24 days by means of an intragastric cannula under light halothane anaesthesia. Each bacterial preparation was used for feeding animals during four consecutive days. Before starting the intervention study, each rat was maintained in the cage for 3 days and afterwards they were monitored daily for weight changes. Additionally, a control group of 8 rats was maintained for 3 days in individual cages and then killed to obtain caecal content at day 0 of intervention. Faeces were collected at 0 (first day of intervention), 4, 11, 18 and 24 days. After 24 days of treatment, the animals were anaesthetized with halothane and, as soon as anaesthesia was assured by loss of pedal and corneal reflexes, they were killed by exsanguination. The liver, spleen, mesenteric lymphoid nodes, small intestine, caecum and

rest of the large intestine were aseptically excised. The caecum-content was also collected. All samples were kept at 4ºC until processing for analyses within the next few hours.

Histopathological evaluation

Samples of the whole intestinal tract were removed, and segments of approximately 2 cm were taken from the duodenum, the midpoint between the bile duct entry and Meckel's diverticulum (jejunum), proximal caecum and rectum. The small intestine and large intestine samples were fixed by immersion in 10% buffered formalin. The samples were then washed overnight with tap water and were dehydrated through a graded series of ethanol. They were incubated in xylene and then embedded in paraffin. Serial sections (5 μm) were taken from all groups and collected onto gelatin-coated slides. Sections were deparaffinized at 60°C overnight, immersed in xylene and rehydrated through a graded series of ethanol. All histological studies were performed on 5 μm sections, stained by haematoxylin and eosin (HE), and examined by light microscope by an experienced pathologist.

Organs, faeces and caecum-content cultivation

The liver, spleen and mesenteric lymphoid nodes as well as the faeces and caecum-content were cultivated immediately after collection. Samples were diluted 1/10 in sterile PBS solution and homogenised for 4 min in a LabBlender 400 stomacher (Seward Medical, London, UK). Serial dilutions were made in Ringer's solution and deep plated into GAM broth (Nissui Pharmaceuticals, Japan) supplemented with 0.25% (w/v) L-cysteine (Sigma) for total cultivable anaerobes counting.

DNA isolation from faeces and caecum-content

DNA was extracted from the homogenised faeces and caecum-content using the QIAamp® DNA stool kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's specifications. For this purpose, an aliquot of 1 mL was centrifuged (10,000 *g*, 15 min, 4ºC) and the pellet was washed twice with PBS and resuspended in the first reagent of the kit.

Quantitative determination of bifidobacteria in the caecum-content and faeces

The quantification of the *Bifidobacterium* genus and the species *B. animalis* and *B. longum* was carried out by qPCR. Previously described primers were used for the genus (Gueimonde et al., 2004b), *B. animalis* (Lahtinen et al., 2005) and *B. longum* (Gueimonde et al., 2006). All reactions were performed in MicroAmp optical plates sealed with MicroAmp optical caps (Applied Biosystems, Foster City, CA, USA) using a 7500 Fast Real Time PCR system (Applied Biosystem) with Sybr green PCR master mix (Applied Biosystems) under conditions previously reported (Salazar et al., 2008).

Qualitative determination of the total microbiota in caecum-content and faeces

The evolution of the microbiota in faeces during the intervention study and the microbiota fingerprint of the caecum-content after the administration period (24 days) were determined by PCR-DGGE. Previously described universal primers (Nübel et al., 1996) were used. The reaction mixture (50 μL) contained 0.25 μmol/L of each primer, 200 μmol/L of each deoxynucleoside triphosphate (Amersham Bioscience, Uppsala Sweden), 2.5 U of *Taq* polymerase (Eppendorf, Hamburg, Germany) and 3 μL of DNA from faeces or caecum content. The amplification program was: 94ºC for 5 min, 35 cycles of 94ºC for 30 s, 58ºC for 20 s, 68ºC for 40 s and the final elongation step of 68ºC for 7 min. The PCR reaction was carried out in an iCycler (BioRad Laboratories, Hercules, CA, USA) apparatus. The PCR products were separated by DGGE in a DCode system (BioRad Laboratories) as described by

234 Salazar and co-workers (2008). Bands were randomly selected to cover variability among rats
235 and study groups. Bands were excised from the gel and used to perform a secondary PCR
236 reaction with the same primers without the GC clamp. After purification, the amplified PCR
237 products were sequenced and partially identified by comparison with data held in the
238 GenBank database (Salazar el al., 2008).
239
240 **Determination of the SCFA in faeces and caecum-content**
241 The analysis of SCFA was carried out by CG-MS as follows. Supernatants from 1 mL
242 of the homogenized faeces and caecum-content samples were obtained by centrifugation
243 (10,000 *g*, 30 min, 4ºC) and filtration (0.45 μm). A chromatographic system composed of a
244 6890N GC (Agilent Technologies Inc., Pal Alto, CA, USA) connected with an ion flame
245 detector and a mass spectrometry 5973N detector (Agilent) was used for quantification as
246 described previously (Salazar et al., 2008).
247
248 **Statistical analysis**
249 Results were analysed using the SPSS v.15 (SPSS Inc. Chicago, USA) software by
250 means of independent one-way ANOVA tests in each sampling point. The differences among
251 the three rat groups were assessed by means of the LSD (least significant difference) mean
252 comparison test ($p<0.05$). To analyse the PCR-DGGE diversity (number of bands), the non-
253 parametric Mann-Whitney test for independent samples was used for pair comparisons
254 between treatment groups.
255
256 **RESULTS**
257 **Viability of bifdobacteria under refrigerated storage and simulated GIT transit**
258 **conditions**

The viability of the bacterial suspensions in milk stored at 4ºC was tested daily for 4 days. The reduction of microbial counts after this time was 0.15±0.14 log units for *B. animalis* IPLA R1 and 1.01±0.27 log units for *B. longum* IPLA E44. The strain R1 remained without noticeable viability loss during cold storage, whereas the viability of E44 rapidly declined after 3 days. Thus, the daily consumption of viable bacteria in rats fed both strains was close to 10^9 cfu per day, except for the 4^{th} day of administration of each E44 preparation (once a week) from which animals received around 10^8 cfu due to the loss of viability of this microorganism during storage.

Survival to the chemically simulated GIT transit of bifidobacteria is depicted in Figure 1. Challenge to GJ pH 3.0 caused similar reduction of counts in both strains (1.15 and 1.54 log units for R1 and E44, respectively). However, in GJ pH 2.0 *B. longum* IPLA E44 population underwent a drastic drop (5.1 log units) whereas *B. animalis* IPLA R1 showed similar counts decrease (1.22 log units) as at pH 3.0. This indicates a lower tolerance of *B. longum* than *B. animalis* to the acidic conditions. The presence of milk in the GJ pH 2.0 increased the pH of the mixture to about 4.0 and counteracted the negative effect of the acid on the strain E44, counts being reduced by only 0.36 log units in these conditions. Regarding the DJ challenge, the strain E44 was also more sensitive to the adverse effects of bile salts (counts reduction of 5.73 log units) than strain R1. The presence of milk in the initial bacterial suspension also protected strain E44 against the subsequent negative effect of bile, the reduction of counts being much lower than without milk. At the end of the sequentially simulated GIT transit, counts of R1 were reduced by about 1.6 log units in all conditions tested, whereas the reduction of the populations of E44 was between 3.5 log units and 5.6 log units, depending on the initial GJ conditions. When using the Live/Dead® BacLight bacterial viability kit after sequential GIT challenge, the number of viable cells was under the detection limit for *B. longum* IPLA E44 (data not shown). For strain R1, viable cells were not found at

initial GJ challenge at pH 2.0, however, the percentage of survival was 57.1±10.1 and 72.6±3.1 for initial GJ pH 3.0 and pH 2.0+milk, respectively ($p<0.05$). These results corroborated the higher resistance to the GIT conditions of strain R1 with respect to strain E44 and the protective effect exerted by milk.

In vitro and *in vivo* safety of bifidobacteria strains

The enzymatic activities and antibiotic MIC values determined for *B. animalis* IPLA R1 and *B. longum* IPLA E44 (Supplementary Material 1). Both strains displayed moderate phosphatase, esterase-lipase and peptidase activities, with the exception of the high activity of leucine arylamidase. The proteolytic activities trypsin and α-chymotrypsin were not detected. The most active glycolytic enzymes were β-galactosidase and α-glucosidase, whereas α-galactosidase, β-glucosidase and N-acetyl-β-glucosaminidase displayed only moderate activity and β-glucuronidase, α-manosidase and α-fucosidase were not detected. Both strains displayed high MIC values for some aminoglycosides (gentamycin, kanamycin, and streptomycin). In addition, tetracycline resistance was found in strain R1(16 μg/mL) but not in strain E44. PCR amplification of genes encoding ribosomal protection proteins and the *tet*(W) gene which confer tetracycline resistance were also positive for R1 but not for E44 strains.

With respect to the *in vivo* experiments of safety, no animal death, abnormal variations in food or water intake, or unexpected behaviour were observed for any animal group during the bifidobacteria intake. In addition, no significant variations ($p > 0.05$) in the animal weight were noticed among the three groups of rats during the treatment [381.0±8.2, 377.0±4.3 and 373.8±7.6 g, for animals fed placebo, *B. animalis* IPLA R1 (B1 group) and *B. longum* IPLA E44 (B2 group) respectively, at 24 days of treatment]. The total anaerobe counts obtained from liver, spleen and mesenteric nodes in the groups of rats fed placebo, R1 and E44 strains

309 (Supplementary Material 2) did not differ significantly ($p > 0.05$) among them for any tissue
310 analyzed, indicating that the oral administration of these microorganisms did not promote
311 bacterial translocation.
312
313 **Histopathological evaluation**
314 No pathological changes were observed macroscopically and microscopically, in
315 either the small intestine or large intestine, for any of the rats analyzed from the control and
316 experimental groups at 0 and 24 days of treatment. No histopathological changes were
317 observed in villi and crypts of small intestine or in mucose of the large intestine in the placebo
318 group and in bifidobacteria-fed rats (group B1 and group B2 respectively) (Figure 2).
319
320 **Quantification of bifidobacteria in the intestinal microbiota of rats**
321 The quantification of total bifidobacteria and the levels of species *B. animalis* and *B.*
322 *longum* in faecal and caecum-content of rats were achieved by qPCR (Figure 3). The
323 evolution of total bifidobacteria in faecal samples at different sampling points during
324 treatment (Figure 3a) showed a similar performance; after an initial increase of about 0.5 log
325 units on day 4, which was probably related to changes in the diet caused by the intake of milk,
326 the counts returned to their initial levels (7.80±0.15 log cfu/g) at the next sampling point (12
327 days). No statistical differences ($p > 0.05$) were found among the three groups of rats at 4, 11
328 and 18 days of feeding whereas *Bifidobacterium* counts were significantly higher ($p < 0.05$)
329 after 24 days in faeces or rats fed either R1 or E44 strains (7.79±0.15 and 7.80±0.19 log cfu/g,
330 respectively) than in faeces of ~~in~~ the placebo group (7.55±0.18 log cfu/g). Regarding the
331 results obtained for the caecum content (Figure 3b), total counts of *Bifidobacterium* genus
332 and counts of *B. animalis* species in the placebo group did not show any significant change at
333 the end of the treatment with respect to the initial values (day 0), whereas counts of *B. longum*

were reduced by 1 log unit. In spite of that, with the only exception of *B. longum* counts in B1 group, levels of total bifidobacteria, *B. animalis* and *B. longum* after 24 days of probiotic administration were significantly ($p< 0.01$) higher in caecum of rats fed bifidobacteria, than in rats fed the placebo. Independent of the species orally administered, treatment with bifidobacteria promoted higher levels of the species *B. animalis* with respect to the placebo group whereas only the administration of the strain *B. longum* IPLA E44 resulted in significantly ($p < 0.05$) higher levels of *B. longum*. Finally, the counts of total bifidobacteria were in the same order of magnitude (from 7.5 to 8.0 log units, depending on the group of rats) in faecal and in caecum-content samples.

Population dynamics of the rat intestinal microbiota

The evolution of total cultivable anaerobes in faecal samples remained without noticeable modifications at the four sampling points taken during the treatment period (data not shown). No significant differences ($p > 0.05$) were detected in faeces and caecum-content among the three groups of rats and the levels found after 24 days of intervention were similar in both types of samples (between 9.03±0.28, and 8.79±0.39 log cfu/g in faeces and between 8.57±0.25, and 8.30±0.44 cfu/g in caecum-content). These results indicate that the oral intake of bifidobacteria didn't have a noticeable effect in the levels of the total cultivable intestinal anaerobic population.

Figure 4 shows the PCR-DGGE fingerprint of the caecal microbial community of four rats per group, and Table 3 shows the identification at species level of some selected bands. A high variability intra- and inter-groups was detected from the patterns of bands, each rat presenting a specific microbiota fingerprint. As a consequence, a pattern related to the oral administration of the two bifidobacterial strains could not be identified. However, the microbial diversity measured as the number of bands (Figure 4b) of the caecum-microbiota,

was significantly higher ($p < 0.001$) in the group of rats fed *B. animalis* IPLA R1 (B1 group) than in rats fed placebo or *B. longum* IPLA E44 strain (B2 group). Most of the 21- selected bands identified (Table 1) corresponded to microorganisms belonging to clostridial XIVa and bands of microorganisms included in clostridial clusters IV and XI were also identified. Similar results were obtained for faecal samples (data no shown) in which, only 3 out of 24 bands identified were lactobacilli from *Lactobacillus johnsonii* (97% similarity) *Lactobacillus murinus / Lactobacillus animalis* (100% similarity) and *Lactobacillus reuteri* (98% similarity) species. In caecum-samples, band 2 (*Ruminococcus gnavus*) was present in all rats analysed and band 19 (*Clostridium citroniae / Clostridium clostridiiformes*) appeared in at least one rat from each group. It is worth mentioning that species considered as butyrate-producers were found in the three groups of rats. This is the case of *Butyrivibrio crossotus* (bands 1 and 3), *Coprococcus eutactus* (band 7), *Clostridium sporosphaeroides* (bands 8 and 12), *Coprococcus catus* (band 13) and *Eubacterium halii* (bands 17 and 21).

Metabolic activity of the intestinal microbiota of rats

The metabolic activity of the intestinal microbiota was determined by measuring the SCFA concentration in faeces and caecum-content samples. The concentration of total and major SCFA (acetate, propionate and butyrate) detected in faeces, increased during the treatment in the three groups of rats with respect to the initial levels (rats at 0 days) (Table 2), although no variation in the proportions of major SCFA was found. The most abundant SCFA in faeces was acetate (around 70%) followed by butyrate and propionate, present in similar proportions (around 17% and 13%, respectively). Regarding the comparison among the three groups of rats, in all sampling points, with the exception of day 18 for B1 group, the concentration of total SCFA was significantly ($p < 0.01$) higher in animals fed bifidobacteria than in the placebo group. However, the proportions of the three major acids remained similar

384 in the three treatment-groups with the exception of results obtained at 4 days of intervention.
385 At this time, a significant ($p < 0.01$) decrease of acetate and a concomitant increase of
386 butyrate were obtained in rats fed *B. longum* IPLA E44. A similar tendency was observed on
387 day 11 in rats fed *B. animalis* IPLA R1. Thus, it seems that the increase of butyrate is
388 correlated with a decrease in acetate levels in the faecal samples at intermediate times during
389 the oral administration of bifidobacteria.

390 The SCFA profile obtained in caecum-content samples (Table 2) was remarkably
391 different from that of faeces. The acetate was also the most abundant SCFA (around 44%),
392 but its proportion was considerably lower than in faecal samples which was in favour of a
393 higher butyrate proportion in caecum than in faeces (around 37%). The propionate remained
394 as the least abundant component (around 18%). Contrary to that found in faeces, comparison
395 among the three groups of rats showed that total and major SCFA concentrations were
396 significantly lower ($p< 0.001$) in rats fed bifidobacteria strains than in the placebo group.

397

398 **DISCUSSION**

399 Several *in vitro* and *in vivo* tests have been proposed by a FAO/WHO experts group
400 (2006) in order to gain insight on strain properties and mechanisms of probiotic effect, as well
401 as on some safety considerations. For bifidobacteria that will be ingested orally included in a
402 probiotic food, survival during the storage of the product, and through the GIT transit, is a
403 desirable property. Suspension in milk at 4ºC for 4 days caused greater viability loses in *B.*
404 *longum* IPLA E44 than in *B. animalis* IPLA R1, although both microorganisms still remained
405 alive for granting a daily intake in rats of about 10^8 cfu. It has been indicated that *B. animalis*
406 species displays good tolerance to oxygen (oxidative stress), thus being able to keep higher
407 counts during manufacture and cold storage of fermented products than other species
408 (Gueimonde et al., 2004a; Jayamanne and Adams, 2009). The suspension of our strains in

409 10%-skimmed milk increased their survival to the chemically simulated GIT passage. In spite
410 of this, strain E44 was considerably less tolerant to the harsh GIT conditions (acid, bile and
411 digestive enzymes) than strain R1. Previous studies indicated that the response and adaptation
412 of *B. animalis* and *B. longum* to different stresses differed between both species (Sánchez et
413 al., 2008). It has been reported that *B. animalis* is more resistant to acidic conditions than *B.*
414 *longum* (Masco et al., 2007) and in the same way we have recently demonstrated that strains
415 of *B. longum,* other than E44, were more sensitive to human gastric and duodenal juices than
416 *B. animalis* strains (de los Reyes-Gavilán et al., 2010). Besides, *B. animalis* IPLA R1 is a
417 bile-adapted strain obtained in our laboratory by exposure of the original sensitive strain to
418 increasing concentrations of bile salts, this probably being another reason for the higher
419 survival in the chemically simulated GIT of this strain with respect to *B. longum* IPLA E44,
420 which is a natural isolate from human faeces (Salazar et al., 2008).

421 Safety is one of the most important criteria for the selection of putative probiotic
422 strains for human consumption. The absence of microbial deleterious metabolic activities,
423 such as those enzymes involved in the transformation of pre-carcinogens into active
424 carcinogens, as well as antibiotic resistance patterns, are recommended (FAO/WHO, 2006).
425 Enzymatic activities such as β-glucosidase and N-acetyl-β-galactosaminidase related with
426 harmful effects in the colon (Parodi, 1999) were detected at low levels in one, or both, of our
427 strains whereas the pro-carcinogenic activivity β–glucuronidase was not present. In addition,
428 E44 and R1 displayed high β-galactosidase activity levels, which have been related with
429 alleviation of symptoms of lactose intolerance. MIC values for most antibiotics were in the
430 range of that described in bifidobacteria (Delgado et al., 2005, 2008). Both microorganisms
431 displayed resistance to aminoglycosides. The intrinsic resistance to aminoglycosides is a
432 common feature in bifidobacteria due to the anaerobic nature of these microorganisms (Mättö
433 et al. 2007) which lack cytochrome-mediated transport (Bryan and Kwan 1981). In addition,

434 *B. animalis* subsp. *lactis* IPLA R1 was resistant to tetracycline, whereas E44 was sensitive. It
435 has been indicated that around 30% of human isolated bifidobacteria are resistant to
436 tetracycline and that dominant species in human adults frequently harbour the *tet*(W) gene
437 responsible for this resistance (Flórez et al., 2006). In our case, the *tet*(W) gene, as well as
438 other genes encoding ribosomal protection proteins were absent in *B. longum* IPLA E44 but
439 they were present in *B. animalis* IPLA R1. In this regard, a recent screening of 26 *B. animalis*
440 strains from a variety of sources revealed the presence of *tet*(W) in all isolates (Gueimonde et
441 al., 2010). A step forward to test the safety of orally delivered probiotic strains is *in vivo* tests
442 using animal models (Huang et al., 2003; Lara-Villoslada et al., 2007; Maragkoudakis, 2009;
443 Tsai et al., 2004; Zhou et al., 2000). The general parameters indicating the well-being status
444 of the Wistar rats used in our study were not affected by the administration of bifidobacteria
445 strains. In addition, bacterial translocation to the liver, spleen and mesenteric nodes induced
446 by the oral administration of the bifidobacteria E44 and R1 strains was not detected, and
447 microbial counts recovered from these organs were in the same range, or even in a lower
448 range, than that reported in literature (Lara-Villoslada et al., 2007; Liong, 2008;
449 Maragkoudakis, 2009). The histological examination of the gut tissues revealed no apparent
450 morphological changes induced by our bifidobacteria. These results support the safety of the
451 strain for oral consumption.

452 Suspensions in 10%- skimmed milk of bifidobacteria were orally administered daily
453 for 24 days to two groups of male Wistar rats and several parameters were analysed and
454 compared against a placebo group not fed bifidobacteria. The qPCR counts of total
455 bifidobacteria population in faeces resulted in significantly higher levels of this genus in the
456 two groups fed either R1 or E44 strains with respect to the placebo group at the end of the
457 treatment. Apart this, in the three groups of rats final levels of faecal bifidobacteria were of
458 the same order as the initial ones and the increase of about 0.5 log units found at the 4th day

459 of intervention may be attributed to diet changes promoted by the administration of milk.
460 Moreover, the oral intake of either *B. animalis* IPLA R1 or *B. longum* IPLA E44 strains led to
461 higher levels of the genus *Bifidobacterium* and of the species *B. animalis* in caecum-content,
462 without promoting noticeable variations in the total viable anaerobic population among the
463 three groups of rats. In contrast, significantly higher levels of the species *B. longum* resulted
464 from the intake of the strain *B. longum* IPLA E44 whereas the administration of *B. animalis*
465 IPLA R1 had no any remarkable effect. It is worth mentioning also the maintenance of *B.*
466 *animalis* levels in the caecum during treatment and the concomitant decrease of about 1 log
467 unit of *B. longum* population in the same period. This suggests that diet modification of rats
468 during our study by administration of skimmed milk to the three designed groups could have
469 allowed the rearrangement of the intestinal bifidobacteria population, favouring the survival
470 of *B. animalis* and impairing permanence of *B. longum*. ~~In a similar way~~ In this respect,
471 Ouwehand and co-workers found in a previous work (2008) that the oral administration of *B.*
472 *animalis* subsp. *lactis* Bb-12 to elderly subjects resulted in faecal increased levels of *B.*
473 *animalis*, but no significant changes in the levels of other bifidobacteria were observed. Some
474 previous studies have also demonstrated that feeding rats with probiotics, prebiotics or
475 synbiotics promoted increases of bifidobacteria population at different locations in the rat GIT
476 (Lesniewska et al., 2006; Montesi et al., 2005; Vasquez et al., 2009). Similar results have
477 been also found in other rodent models (Kumar et al., 2008; Plant et al., 2003). Total
478 bifidobacteria counts obtained in our study by using qPCR were in the same order of that
479 reported for rat faecal samples by other authors (Delroisse et al., 2008). In this respect,
480 Montesi and co-workers (2005) found that *B. animalis* is the most abundant species in Wistar
481 rats whereas Vasquez and co-authors (2009) indicated that is *Bifidobacterium pseudolongum*.
482 On the other hand, the PCR-DGGE analysis of microbial populations in caecum-content
483 showed that rats fed *B. animalis* IPLA R1 presented a higher diversity of bands than the

placebo group, or the group of rats fed *B. longum* IPLA E44. These differences in the effect promoted by both microorganisms could be related to their different survival rates, different intrinsic characteristics of each strain, and/or to the influence of the administration of skimmed milk together with probiotics in the dynamics of the different bifidobacterial intestinal populations. In this way, other authors have noticed an increase in the number of PCR amplification products subsequent to probiotic / prebiotic treatments (Licht et al., 2006; Montesi et al., 2005; Sarmiento-Rubiano et al., 2007). In our work we have not been able to associate a bacterial fingerprint with the oral administration of a given bifidobacteria strain; this is probably due to the complexity of the rat microbiota which is unique for each individual, as has also been reported for the human microbiota (Favier et al., 2002; Vanhoutte et al., 2004). Amplification and sequencing of several bands of PCR-DGGE gels allowed us to identify microorganisms belonging to clostridial clusters XIVa, XI, and IV. Although lactobacilli have been identified in faecal samples they were not found in the caecum-content. No other bacterial groups, usually found in the gut microbiota of rats such as bacteroides or enterobacteria (Lesniewska et al., 2006; Licht et al., 2006; Montesi et al., 2005), were identified, which could be partly attributed to limitations of primers and experimental conditions used in the present work for PCR amplifications (Vanhoutte et al., 2004).

SCFA are metabolites produced by the intestinal microbiota and they play an important role by maintaining a healthy colonic environment (Wong et al., 2006). The amount of total SCFA as well as acetate, propionate and butyrate increased during the treatment in our placebo and bifidobacteria-treated groups which could be due to the introduction of skimmed milk acting as prebiotic in the diet of rats. The total SCFA concentration in our placebo group was close to the range reported in literature for Wistar rats (Juskiewicz et al., 2007; Sarmiento-Rubiano et al., 2007). Notably, the total SCFA concentration was significantly lower in the caecum-content, and higher in the faecal samples of bifidobacteria-fed groups,

509 with respect to that of the placebo group. This suggests that the production of SCFA is
510 probably being enhanced in parts of the colon beyond the caecum in rats fed bifidobacteria.
511 Proportions of acetate and butyrate were of the same order in caecum-content samples,
512 whereas in faeces the acetate was dominant over butyrate. This fact was attributed not only to
513 higher absolute concentrations of acetate, but also to lower absolute concentrations of
514 butyrate. The lower level of butyrate in faeces with respect to the caecum could be explained
515 by a fast consumption of this SCFA by the epithelial intestinal cells of the distal intestine. The
516 higher level of acetate in faeces suggests that the microbiota located between the caecum and
517 distal intestine was actively producing this metabolite. This fact agrees with the enhancement
518 of SCFA production found by us in distal parts of the intestine when bifidobacteria were
519 orally administered.

520 In conclusion, *in vitro* and *in vivo* studies corroborated the safety of strains *B. animalis*
521 IPLA R1 and *B. longum* IPLA E44 and the better survival of R1 than E44 to refrigerated
522 storage and to the GIT transit. The continuous administration of both strains to Wistar rats
523 promoted significant changes in the population of bifidobacteria and in the SCFA
524 concentration of faeces and caecum-content without affecting total anaerobic counts and
525 proportions of different major SCFA, thus contributing to modulate the intestinal microbiota
526 of rats.

527

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688

689 **FIGURE LEGENDS**

690 **Figure 1:** Survival to the chemically simulated GIT of *B. animalis* subsp. *lactis* IPLA R1 and
691 *B. longum* subsp. *longum* IPLA E44. Counts (log cfu/mL) were obtained at each sampling
692 point (a) and the percentage of survival by using fluorescence probes (Live/Dead® BacLigth,
693 Invitrogen) was measured at the end of the GIT transit (b). Simulated GIT transit steps, GJ:
694 gastric juice, DJ: duodenal juice and IJ: intestinal juice. GJ pH 2.0 (●), GJ pH 3.0 (□) and GJ
695 pH 2.0 + 10%-skimmed milk (▲). The coefficient of variation (100* SD/mean) of these data
696 ranged between 1.1 – 2.3% (a) and 4.3 – 17.7% (b). At the end of the GIT challenge, the
697 symbols/bars that do not share a common letter are significantly ($p<0.05$) different according
698 to the mean comparison test LSD.

699

700 **Figure 2:** Photomicrographs of small and large intestine sections stained with haematoxylin-
701 eosin in control rats at 0 days (a), rats fed with placebo after 24 days (b), rats fed with
702 bacterial suspensions in milk (10^9 cfu/day) of *B. animalis* subsp. *lactis* IPLA R1 (B1 group)
703 after 24 days (c), and of *B. longum* subsp. *longum* IPLA E44 (B2 group) after 24 days (d).
704 Crypts are indicated by filled arrows and villi are indicated by dashed arrows.

705

706 **Figure 3**: Quantitative PCR counts (log nº cells /gram) of the *Bifidobacterium* genus in faecal
707 samples at different sampling points (a) and of genus *Bifidobacterium* and species *B. animalis*
708 and *B. longum* (detection limit 4.0, 4.0 and 4.6 log cfu/mL, respectively) in caecum-content
709 samples at 0 and after 24 days of treatment (b) in three groups of rats fed placebo (skimmed
710 milk, black symbol) or bacterial suspensions in milk (10^9 cfu/day) of *B. animalis* subsp. *lactis*
711 IPLA R1 (group B1, grey symbol) and *B. longum* subsp. *longum* IPLA E44 (group B2, white
712 symbol). The coefficient of variation (100* SD/mean) of these data ranged between 1.9 and

713 3.7% in graphic (a). For each sampling point, results that do not share a common letter are
714 significantly ($p<0.05$) different according to the mean comparison test LSD.
715
716 **Figure 4**: PCR-DGGE profiles (a) of caecum-content samples obtained after 24 days from
717 four rats (R1, R2, R3 and R4) of each group tested: rats fed with placebo (P), with bacterial
718 suspensions in milk (10^9 cfu/day) of *B. animalis* subsp. *lactis* IPLA R1 (B1) and *B. longum*
719 subsp. *longum* IPLA E44 (B2). Numbers inside the gels refer to sequenced DNA bands,
720 whose tentative identification is indicated in Table 1. "Box and whiskers" representation of
721 the PCR-DGGE diversity (number of bands) analysed in eight rats per treatment group (b);
722 boxes that do not share a common letter are significantly different ($p<0.001$) accordingly to
723 the non-parametric Mann-Whitney test.
724
725