Exopolysaccharides produced by *Bifidobacterium longum* IPLA E44 and *Bifidobacterium animalis* subsp. *lactis* IPLA R1 modify the composition and metabolic activity of human faecal microbiota in pH-controlled batch cultures

Running title: *Bifidobacterium* EPS modify human faecal microbiota composition

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ABSTRACT

Exopolysaccharides (EPS) isolated from two *Bifidobacterium* strains, one of human intestinal origin (*Bifidobacterium longum* subsp. *longum* IPLA E44) and the other from dairy origin (*Bifidobacterium animalis* subsp. *lactis* IPLA R1), were subjected to *in vitro* chemically simulated gastrointestinal digestion, which showed the absence of degradation of both polymers in these conditions. Polymers were then used as carbon sources in pH-controlled faecal batch cultures and compared with the non-prebiotic carbohydrate glucose and the prebiotic inulin to determine changes in the composition of faecal bacteria. A set of eight fluorescent *in situ* hybridisation oligonucleotide probes targeting 16S rRNA sequences were used to quantify specific groups of microorganisms. Growth of the opportunistic pathogen *Clostridium histolyticum* occurred with all carbohydrates tested similarly to that found in negative control cultures without added carbohydrate and was mainly attributed to the culture conditions used rather than enhancement of growth by these substrates. Polymers E44 and R1 stimulated growth of *Lactobacillus* / *Enterococcus*, *Bifidobacterium*, and *Bacteroides* / *Prevotella* in a similar way to that seen with inulin. The EPS R1 also promoted growth of the *Atopobium* cluster during the first 24 h of fermentation. An increase in acetic and lactic acids was found during early stages of fermentation (first 10-24 h) correlating with increases of *Lactobacillus*, *Bifidobacterium*, and *Atopobium*. Propionic acid concentrations increased in old cultures, which was coincident with the enrichment of *Clostridium* cluster IX in cultures with EPS R1 and with the increases in *Bacteroides* in cultures with both microbial EPS (R1 and E44) and inulin. The lowest acetic to propionic acids ratio was obtained for EPS E44. None of the carbohydrates tested supported the growth of microorganisms from *Clostridium* clusters XIVa+b and IV, results that correlated with the poor butyrate production in the presence of EPS. Thus, EPS synthesized by bifidobacteria from dairy and intestinal origins can modulate the intestinal microbiota *in vitro*, promoting

52 changes in some numerically and metabolically relevant microbial populations and shifts in the
53 production of short chain fatty acids.
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INTRODUCTION

The human colonic microbiota is a complex ecosystem dominated by obligate anaerobes that can reach levels of over 10^{11} cells per gram of contents (Bäckhed et al., 2005). Dietary compounds not digested by the host are the main carbon source from which gut microorganisms obtain energy. These substrates, which mainly include plant oligo and polysaccharides, can be subjected to gut bacterial metabolic transformations and hence they can have a major influence on human nutrition and health (Flint et al., 2008). Short chain fatty acids (SCFA) and lactate are the main products arising from the microbial fermentation of carbohydrates. SCFA can provide energy to the colonic epithelium, modulate cholesterol and lipid metabolism, suppress pathogenic intestinal bacteria and modulate the immune system (Cummings and Macfarlane, 1997; Topping and Clifton, 2001). Chemical composition and availability of fermentable carbohydrates in the colon have a strong influence on the gut microbiota composition. Specific carbohydrates are now being used as prebiotics based on the concept that they stimulate selected beneficial bacteria in the colon (Gibson and Roberfroid, 1995). The most studied prebiotics are inulin and fructooligosaccharides (FOS) (Roberfroid, 2007), and they have a recognised bifidogenic effect (Bouhnik et al., 2004; Gibson et al., 1995; Kruse et al., 1999; Rossi et al., 2005).

Many strains of lactic acid bacteria and bifidobacteria are able to produce exopolysaccharides (EPS). The physiological functions of these carbohydrate polymers have not yet been clearly determined (Ruas-Madiedo et al., 2008). Among the beneficial effects attributed to these biopolymers such as cholesterol lowering capability and immunomodulating ability, the possibility of acting as fermentable substrates *in vitro* has been demonstrated to date by Korakli and coworkers (Korakli et al., 2002) for a fructan-type EPS produced by a strain of *Lactobacillus sanfranciscensis*. There was also evidence of an *in vitro* bifidogenic effect for the levan-type EPS produced by another strain of the same species (Dal Bello et al., 2001). We have recently

demonstrated in a non pH-controlled faecal slurry culture that EPS isolated from different human intestinal bifidobacteria can act as fermentable substrates for microbial intestinal populations (Salazar et al., 2008). This promoted shifts in SCFA profiles, moderate increases in bifidobacteria populations, and changes in banding patterns when cultures were analysed by PCR-DGGE using universal primers, which indicated microbial population shifts.

Bifidobacteria are intestinal microorganisms that are being extensively used as probiotics, based on health-promoting benefits attributed to them (Masco et al., 2005). In order to gain further insight into the changes that EPS from bifidobacteria may mediate in the intestinal microbiota, in the present work we quantified modifications in the levels of selected microbial groups by fluorescent in situ hybridisation (FISH) and established possible relationships between metabolic changes and variations in microbial populations by using pH-controlled faecal batch cultures. Compared to our previous non pH-controlled experiments (Salazar et al., 2008), this design is a more realistic simulation of the conditions in the human distal colon with regards to local pH, substrate availability and transit time, as the semisolid luminal contents of the distal colon are almost static therein. Two microbial polymers were used in this study as carbon sources; EPS E44 was produced by a human faecal isolate of *Bifidobacterium longum* subsp. *longum*, one of the most abundant species of *Bifidobacterium* found in the faecal samples of donors from our region (Asturias, North of Spain) (Delgado et al., 2006). This EPS was chosen on the basis of the high reduction of acetic to propionic acid ratio promoted by polymers of the species *B. longum* and particularly by E44 (Salazar et al., 2008). The EPS R1 was produced by a strain of *Bifidobacterium animalis* subsp. *lactis*, species widely used as adjunct culture in functional dairy products. Cultures of *B. animalis* subsp. *lactis* IPLA R1 present a ropy phenotype which could be an interesting technological property (Salazar et al., 2009). Both strains were considered in order to ascertain if the origin of the EPS producing strain and their different monosaccharide

composition could influence microbiota dynamics. The use of bifidobacteria producing fermentable EPS may have the advantage over other known prebiotic substrates of the use / administration of both, a potential probiotic and a fermentable substrate, in a single step.

MATERIAL AND METHODS

EPS-producing strains and culture conditions

B. longum subsp. *longum* IPLA E44 was previously isolated from faeces of a healthy adult volunteer with the approval of the Regional Ethics Committee (Asturias, Spain; Delgado et al., 2006). *B. animalis* subsp. *lactis* IPLA R1 was of dairy origin (Ruas-Madiedo et al., 2006). Both strains are held in the IPLA's culture collection. Strains identity was confirmed by partial amplification of the 16S rRNA gene using primers Y1-Y2 (Young et al., 1991), sequencing and alignment with sequences from reference strains held in the GenBank database. Bifidobacteria were grown in MRSC broth (MRS broth [Difco, Becton Dickinson, MD, USA] supplemented with 0.25% [w/v] L-cysteine [Sigma Chemical Co., St. Louis, MO]) and were incubated at 37°C for 24 h in an anaerobic cabinet (Mac 1000; Don Whitley Scientific, West Yorkshire, UK) under a 10% H₂, 10% CO₂, and 80% N₂ atmosphere.

EPS isolation

EPS fractions produced by *Bifidobacterium* strains were isolated from the cellular biomass harvested from MRSC agar plates (Ruas-Madiedo et al., 2006). Bacteria were grown by creating a culture lawn with sterile glass beads on the surface of MRSC agar plates that were then incubated under anaerobic conditions at 37°C for 5 days. Cell biomass was collected using 2 mL ultrapure water per plate and the resulting volume mixed with 1 vol of 2 M NaOH. The suspension was gently stirred overnight at room temperature to promote polymer release from the cellular surface.

Cells were removed by centrifugation at 8,400 x g for 30 min and EPS was precipitated from the supernatants over 48 h at 4°C using 2 vol of cold absolute ethanol. Following centrifugation, the EPS fraction was resuspended in ultrapure water and dialysed for 3 days at 4°C with daily changes of water, using dialysis tubes (Sigma) of 12 to 14 kDa molecular mass cut off. Finally, the dialysed EPS fractions were freeze-dried.

Chemical simulation of the EPS gastrointestinal transit

The digestibility of EPS through the gastrointestinal tract was studied using an *in vitro* model that chemically simulates the physiological conditions. The simulated gastric juice contained 125 mM NaCl, 7 mM KCl, 45 mM NaHCO₃, and 3 g/L pepsin (Sigma) pH 2.0 adjusted with HCl, and the simulated intestinal juice was composed of 0.3% (w/v) bovine bile, and 0.1% (w/v) pancreatin (Sigma) pH 8.0 adjusted with 10 N NaOH. The EPS fractions were weighted and dissolved at a concentration of 10 mg/mL in ultra-pure water. Two different simulations were carried out independently, one mimicking gastric digestion and the other simulating small intestine digestion. In both cases 100 µL EPS solution were mixed with 900 µL of either gastric or intestinal juice for obtaining a final concentration in the experiment of 1 mg/mL EPS, which has been the lowest dose tested by us in a previous work having any effect (Ruas-Madiedo et al., 2006). The suspension in gastric juice was incubated for 90 min at 37°C with a mild stirring and finally the pH was brought to 4.5-5.0 with 10 N NaOH, and the suspension in intestinal juice was incubated for 120 min at 37°C in anaerobic conditions. Samples were then centrifuged (10,000 x g) for 10 min and supernatants collected for size exclusion chromatography (SEC) analyses.

A HPLC chromatographic system composed of an Alliance 2690 module injector, a Photodiode Array PDA 996 detector, a 410 refractive index (RI) detector and the Empower

software (Waters, Milford, MA, USA) was used. Additionally, a multi-angle laser light scattering detector (MALLS) (Dawn Heleos II, Wyatt Europe GmbH, Dembach, Germany) was coupled in series. The sample separation (50 μ L vol injection) was carried out in two SEC columns placed in series, TSK-Gel G3000 PW_{XL} + TSK-Gel G5000 PW_{XL} protected with a TSK-Gel guard column (Supelco-Sigma). The mobile phase was 0.1 M NaNO₃ and the separations took place at 40°C at a flow rate of 0.45 mL/min. The EPS peaks were detected and quantified with the RI detector. Standards of dextran (Fluka-Sigma), ranging from 5x10³ to 4.9x10⁶ Da, were used for EPS quantification. The molar mass (Mw) was determined by using the MALLS detector. Assays were carried out in triplicate and HPLC runs were performed in duplicate.

Faecal sample preparation

Faecal samples were obtained from three healthy adult donors (two men and one woman, aged 31 to 40 years) who were free from known metabolic and gastrointestinal disorders. None of the volunteers had taken antibiotics during the 6 months leading to the study. Samples were collected, kept in anaerobic cabinet and used within a maximum of 15 min after collection. A 1/10 w/v dilution in pre-reduced phosphate-buffered saline solution (PBS: 8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na₂HPO₄, and 0.2 g/L KH₂PO₄) pH 7.3 was prepared and homogenised in a stomacher (Seward, UK) for 2 min.

Batch culture fermentations

Five gently stirred pH-controlled batch fermenter (80 mL working volume) were run in parallel, in which each of the following carbohydrates were added: EPS isolated from the strains *B. animalis* IPLA R1 and *B. longum* IPLA E44, inulin (Beneo™ ST, Orafti, Tienen, Belgium; low

molecular weight inulin > 90%, average DP $\geq$ 10, glucose + fructose $\leq$ 4%, sucrose $\leq$ 8%) or glucose (non-selective control). A control with no carbohydrate added (negative control) was also included. The experiment was performed in triplicate, using one faecal sample given by a different donor for each run of five batch fermenters. Eighteen mL of sterile basal medium (BM) were aseptically added to each fermenter. Briefly, BM contained 2 g/L peptone water (Oxoid Ltd., Basingstoke, UK), 2 g/L yeast extract (Oxoid), 0.1 g/L NaCl (BDH Chemical Ltd., Dorset, UK), 0.04 g/L KH_2PO_4 (BDH), 0.04 g/L K_2HPO_4 (BDH), 0.01 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (BDH), 0.01 g/L $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ (BDH), 2 g/L NaHCO_3 (BDH), 2 mL Tween-80 (BDH), 0.05 g/L haemin solution (Sigma), 10 μL Vitamin K_1 (Sigma), 0.5 g/L L-cysteine (Sigma) HCl, 0.5 g/L bile salts (Oxoid), and 4 mL resazurin 0.025 % w/v as an anaerobic indicator. The fermenters were sparged overnight with O_2 -free N_2 at 15 mL/min.

The test carbohydrates (1% w/v) were added to each vessel just before the addition of 2 mL (10% v/v) of faecal slurry prepared as described above. The temperature was kept at 37°C by means of a circulating water bath and the pH was maintained between 6.7 and 6.9 using an Electrolab pH controller (Tewkesbury, UK). The batch cultures were run for a period of 72 h and 2 mL samples were obtained from each vessel at 0, 10, 24, 34, and 48 h for FISH and SCFA analyses. A further sample was obtained at 72 h for SCFA analyses.

Enumeration of bacteria in faecal cultures using FISH

The FISH technique was used to quantify selected bacterial groups of the gut microbiota following the method described by Daims et al. (2005). Samples (375 μL) obtained from each vessel at each sampling time were fixed for 6 min at 4°C with 1.125 mL of 4% (w/v) filtered paraformaldehyde (pH 7.2). Fixed cells were centrifuged at 15,000 x g for 5 min and washed

twice in filtered PBS. The washed cells were finally suspended in a mixture of 150 μ L filtered PBS and 150 μ L (99%) ethanol and stored at -20°C for at least 1 h before further processing. The samples were then diluted in a suitable volume of filtered PBS in order to obtain 10-100 fluorescent cells in each microscope field of view and 20 μ L of the above solution was added to each well of a 6 well Teflon/poly-L-lysine coated slide (Tekdon Ins., Myakka City, USA). The samples were dried for 15 min in a drying chamber (46°C). They were then dehydrated using an alcohol washing series (50, 80 and 96% [v/v] ethanol) for 3 min with each solution. Slides were returned to the drying oven for 2 min to evaporate the excess ethanol before adding the hybridisation mixture. Lab158 probe required a pre-treatment with 20 μ L lysozyme (1 mg/mL) at 37°C for 15 min. Following incubation the slides were washed briefly (2-3 s) in distilled water to remove the lysozyme solution and were then dehydrated in the ethanol series as described above.

Synthetic oligonucleotide probes targeting specific regions of the 16S rRNA gene labelled with the fluorescent dye Cy3 (Sigma), were utilized for the enumeration of the *Bifidobacterium* genus, *Bacteroides/Prevotella* group, *Lactobacillus/Enterococcus* group, *Clostridium coccoides/Eubacterium rectale* group (*Clostridium* clusters XIVa + b), *Clostridium histolyticum* subgroup (clusters I and II), *Clostridium* cluster IX, *Faecalibacterium prausnitzii* and relatives (part of *Clostridium* cluster IV), and *Atopobium* cluster (Table 1). For the enumeration of total cells, samples were stained with the nucleic acid stain 4', 6-diamidino -2-phenylindole (DAPI).

50 μ L of the hybridisation mixture (5 μ L of each probe [50 ng/ μ L] and 45 μ L of filtered hybridisation buffer [0.9 M NaCl, 20 mM Tris-HCl pH 8.0, 0.01% SDS w/v]) were added to each well and allowed to hybridise for 4 h in a microarray hybridisation incubator (Grant Boekel, Cambridge, UK). Following hybridisation, slides were washed in 50 mL of filtered washing buffer (0.9 M NaCl, 20 mM Tris-HCl pH 8.0) for 15 min. They were then dipped in cold water for

a few seconds and dried with compressed air. 5 μ L of antifade reagent (polyvinyl alcohol mounting medium with DABCOTM antifading, Sigma) was added to each well and a coverslip was applied (20 mm coverslips, thickness n° 1, VWR, Lutterworth, UK). Slides were stored in the dark at 4°C for a maximum of 3 days and during this time were examined using an epifluorescence microscope Nikon Eclipse 400 (Nikon, Surrey, UK) using the Fluor 100 lens. Filters for the DAPI stain (excitation at 550 nm and emission at 461 nm) and the Cy3 dye (excitation at 550 nm and emission at 564 nm) were used. A minimum of 15 fields of view were counted for each sample. Numbers of specific bacteria and DAPI-stained entities (used to count total bacteria) were determined using the following equation:

$$DF \times ACC \times 6732.42 \times 50 \times DF_{\text{sample}}$$

where DF is the dilution factor ($300/375 = 0.8$) and ACC is the average cell count of 15 fields of view. The figure 6732.42 refers to the area of the well divided by the area of the field of view. The factor 50 takes the cell count back to per millilitre of sample and DF_{sample} refers to the dilution of sample used with a particular probe or stain.

Analysis of SCFA and lactic acid

Samples (1 mL) were centrifuged twice ($17,000 \times g$) for 15 min to remove cells and particulate materials. The supernatant (20 μ L) was injected into a HPLC system (Hewlett-Packard HP 1050 series) equipped with a UV detector set at 210 and an automatic injector. An ion-exclusion REZEX-ROA organic acid column (Phenomenex Inc., Macclesfield, UK) was used, which was maintained at 83.2°C with a column heater. The eluent, 0.0025 M sulphuric acid in HPLC-grade water, was pumped through the column at a flow rate of 0.5 mL/min. Data from the UV detector were integrated using the ValueChromTM software package (BioRad). The

concentration (mM) of each SCFA and lactate was calculated using linear regression equations ($R^2 \geq 0.99$) from the corresponding curves of standards obtained with six different concentrations. Total SCFA concentrations were calculated as the sum of the three major SCFA (acetic acid plus propionic acid plus butyric acid). The percent of total SCFA for each of the three major SCFA was also calculated from total SCFA concentration and the concentration of the corresponding SCFA.

Statistical analysis

Statistical analysis was performed using the SPSS-PC 11.0 software (SPSS Inc., Chicago, Illinois, USA). Data of simulated gastrointestinal digestion were subjected to a one-way ANOVA. For the FISH data, growth of microbial groups targeted by each probe was evaluated by a one-way ANOVA in order to determine whether there were significant differences at a given fermentation time among the substrates evaluated by using “carbohydrate” as a factor with five categories (negative control, EPS R1, EPS E44, inulin, and glucose); where appropriate, the least significant difference test (LSD) was used for comparison of means. Another One-way ANOVA and LSD test were performed in order to determine whether there was any significant difference among times of fermentation for each specific carbohydrate by using “time of incubation” as factor with five categories (0, 10, 24, 34, and 48 h).

RESULTS

Susceptibility of EPS to degradation under simulated gastrointestinal conditions

Susceptibility of the EPS E44 and R1 to simulated gastric and intestinal juices was evaluated by possible modifications on the amount of EPS recovered before and after submission

to treatments. The amount of the highest peak of both EPS fractions ($7.4 \pm 2.50 \times 10^5$ g/mol and $1.55 \pm 0.20 \times 10^6$ g/mol, for E44 and R1, respectively) recovered from SEC did not show significant variation after any treatment (Fig. 1). Thereby, E44 and R1 were not degraded during submission to simulated gastric and intestinal conditions.

Changes in faecal populations by FISH analysis

In vitro modification of the faecal microbiota by EPS, inulin and glucose was monitored by FISH (Table 2. In the negative control cultures, most bacterial groups remained without significant variation throughout fermentation. The exception was *Atopobium* cluster and *C. histolyticum* group for which a significant decrease and increase ($P < 0.05$) was observed, respectively, at 48 h and from 10 h of incubation with respect to 0 h. In contrast, the addition of carbohydrates led to more noticeable changes in the faecal microbiota. The addition of glucose and the EPS R1 and E44 promoted an increase of total bacteria during incubation (from 10 h of fermentation with respect to 0 h; $P < 0.05$). Relating the specific microbial groups, *Lactobacillus* / *Enterococcus*, *Bifidobacterium*, and *Bacteroides* / *Prevotella* showed a similar behaviour with all carbohydrates tested. Therefore, they were the only bacterial groups out of the eight enumerated that increased significantly ($P < 0.05$) during batch culture fermentations (from 10, 24 and 48 h of incubation for each of the three groups, respectively) with inulin, glucose and with the EPS isolated from *B. animalis* IPLA R1 and *B. longum* IPLA E44 as compared to the negative control cultures. However, probably because of the variations between donors, the comparison with 0 h within the same culture did not render significant differences through fermentation (10, 24, 34, and 48 h of incubation) for *Bifidobacterium* with EPS E44 and for *Bacteroides* / *Prevotella* with R1.

Clostridium cluster IX and *Atopobium* group displayed different behaviour depending on the carbohydrate. Thus, the *Clostridium* cluster IX became enriched from 10 to 48 h in cultures with glucose and with the EPS R1 compared with 0 h ($P < 0.01$). Polymer R1 also promoted growth of the *Atopobium* group at 10 and 24 h of incubation with respect to time 0 ($P < 0.05$). This group of microorganisms decreased from this point to the end of fermentation (48 h) in a similar way to that of the negative control ($P < 0.05$). Remarkably, neither EPS E44 nor inulin had any effect on *Clostridium* cluster IX and *Atopobium*. *C. histolyticum* group displayed significant increases up to 48 h of fermentation with respect to 0 h with all test carbohydrates ($P < 0.05$) as it was also found in negative control cultures ($P < 0.001$). Finally, no major differences were seen in *Clostridium* cluster XIVa+b, and the *F. prausnitzii* group when assessing the effect of inulin, glucose and the two microbial EPS during incubation, either when the test carbohydrates were compared to each other or to the negative controls.

SCFA and lactate production in batch cultures

Marked differences were found among donors with respect to levels of SCFA and lactate attained in faecal cultures. Because of this, data are presented separately for each donor and a statistical analysis was not carried out. The production of lactic acid was higher in the presence of the test carbohydrates compared to the negative control cultures, which indicated that carbohydrates were actively fermented by the faecal microbiota (Table 3). Lactic acid production in faecal cultures from individuals 2 and 3 occurred during early stages of fermentation and maximum levels were achieved at 10 h, with the exception of donor 2 with inulin. In faecal cultures from donor 1, the highest lactic acid production occurred later (48 h of incubation) for EPS E44 and inulin. The fermentation of EPS E44 gave rise to more lactic acid production during incubations than EPS R1 (maximum values attained during fermentation by cultures from

different donors ranged from 3.63 to 16.74 mM and from 2.29 to 3.30 mM for E44 and R1, respectively), probably indicating a more efficient utilisation of the former polymer by the intestinal microbiota.

In general, total SCFA increased during faecal batch culture fermentation in both the presence and absence of the test substrates (Table 4). However, this increase was considerably more pronounced in the presence of carbohydrates than in negative controls, thus stimulating SCFA production from the external carbon sources. Acetic acid (~~Table 4~~) was the most abundant SCFA, followed by propionic acid and considerable lower amounts of butyric acid (Table 4). Although in general the absolute concentration of each of the three major SCFAs increased during incubation (data not shown) with respect to time 0, the percent of acetic acid with each carbohydrate decreased in most cases from 10 to 72 h of incubation and the contrary occurred for propionic acid, whose percent seems to increase throughout the fermentations. When absolute molar concentrations of propionic acid in the test substrate cultures and in different donors were considered, it appears that a higher production of this acid at 72 h of incubation with respect to 0 h occurred in the presence of the EPS E44 than with the other carbon sources tested (Table 3). The contrary was found for glucose which resulted in the lowest increase of propionic acid. As a consequence of the SCFA production pattern indicated above, the acetic acid to propionic acid ratio decreased from early stages (0-10 h) to the end of incubation (72 h) (Table 4). Notably, the lowest values of this ratio among the carbohydrates tested were obtained in cultures with EPS E44 and the highest in cultures with glucose. Finally, butyric acid production differed among carbohydrates, reaching the highest levels in the presence of inulin rather than in the presence of microbial EPS.

DISCUSSION

We recently demonstrated that EPS isolated from bifidobacteria of human origin could act as fermentable substrates for intestinal bacteria and qualitative rearrangements of several microbial populations were evidenced by PCR-DGGE (Salazar et al., 2008). However, EPS could only act as fermentable substrates for colonic bacteria if they arrive not degraded to the colon. Under the conditions used in our study, EPS E44 and R1 did not suffer an *in vitro* breakdown by simulated gastric and intestinal juices. This means that long molecules ($M_w > 10^6$ g/mol) may still remain available for fermentation in the colon if they are ingested, as could be the case of the polymer R1 which is synthesized by a strain of dairy origin. To this respect, other authors indicated that EPS from lactic acid bacteria can be either partially broken down (Mozzi et al., 2009) or be resistant to the harsh conditions of gastrointestinal transit (Looijesteijn et al., 2001). Then, we used FISH to monitor quantitative changes in the composition of certain numerically predominant faecal microbial groups in pH-controlled batch cultures from faeces and EPS. Our results were compared with inulin and glucose, used as positive selective and non-selective controls, respectively. Glucose is not abundant in the colon and presumably its influence in the ecological intestinal ecosystem would be negligible. However, complex polymers such as inulin can be present in the diet reaching the colon undigested.

Polymers produced by *B. animalis* subsp. *lactis* IPLA R1 and *B. longum* subsp. *longum* IPLA E44 are heteropolysaccharides as indicated in previous studies (Ruas-Madiedo et al., 2007). The content of monosaccharides differed between both polymers, with EPS R1 composed of rhamnose, galactose and glucose whereas E44 contained glucose and galactose, but not rhamnose. Since different microbial glycosidases and mono and oligosaccharides cell-transport systems are needed for the use of polysaccharides, it could be assumed that differences in composition of both polymers could account for some of the differences observed in the response of the faecal microbiota in the presence of EPS in the present work.

The presence of both microbial EPS tested in this work promoted an increase of total bacteria during fermentation. An increase in *C. histolyticum* occurred in the presence of all carbohydrates tested including in negative controls and as such could be attributed to factors such as culture conditions rather than to a specific effect mediated by the test substrates. Among the other groups analysed by FISH, E44 and R1 clearly stimulated the growth of *Lactobacillus* / *Enterococcus* and the genus *Bifidobacterium*, which corroborated our previous findings with regards to the increase of bifidobacteria population by quantitative RT-PCR (Salazar et al., 2008). Bifidobacteria and lactobacilli have been traditionally considered as the main microbial targets of prebiotic action (Gibson and Roberfroid, 1995; Reid, 2008). At this point it is necessary to take into account that no a totally specific probe targeting most species of *Lactobacillus* genus but discarding enterococci is currently available; in spite of that, Lab 158 was chosen for the present work on the basis of its good performance with common intestinal lactobacilli (Harmsen et al., 1999). Therefore, as the Lab158 probe enumerates both lactobacilli and enterococci (Harmsen et al., 1999), a concomitant increase of enterococci should not be from our results. Inulin and inulin-derived substrates have previously been reported by other authors to enhance the growth of bifidobacteria and lactobacilli (Hopkins and Macfarlane, 2003; Wang and Gibson, 1993). Here also, both inulin and glucose stimulated growth of *Lactobacillus* / *Enterococcus* and *Bifidobacterium* groups. To date, the prebiotic effect of EPS produced by lactic acid bacteria has only been demonstrated for two fructan-type homopolysaccharides produced by *Lactobacillus sanfranciscensis* (Dal Bello et al., 2001; Korakli et al., 2002). To the best of our knowledge, polymers R1 and E44 are the first heteropolysaccharides for which a stimulatory effect on intestinal bifidobacteria and lactobacilli populations has been documented.

Bifidobacteria, lactobacilli and some microorganisms of the *Atopobium* cluster (Dewhirst et al., 2001; Downes et al., 2001) are lactic acid producers; acetic acid is formed by many

anaerobic bacteria from the human gut (Macfarlane and Gibson, 1997), also being a principal end product of carbohydrate fermentation by bifidobacteria. In faecal cultures with EPS, inulin and glucose added, the rapid increase in acetic and lactic acids during early stages of fermentation (until 10 h and in certain cases until 24 h of fermentation) correlated well with the increase of total bacterial counts as well as with increases in lactobacilli and bifidobacteria together with increases in *Atopobium* with R1 occurring at those times. The production of lactic and acetic acids by the groups of microorganisms commented above (lactobacilli, bifidobacteria and *Atopobium* cluster) could account for metabolic cross-feeding mechanisms that could probably occur among lactic acid bacteria and other faecal microbial groups (Salazar et al., 2008).

Bacteroides and *Prevotella* are among the predominant genera in the gut of mammals (Hooper et al., 2002). They are versatile saccharolytic microorganisms and the major part of polysaccharide digestion that occurs in the human large intestine is carried out by members of the genus *Bacteroides* (Salyers, 1984). Therefore, it can be assumed that they could play a key role in the degradation of the carbohydrate sources used in pH-controlled fermentations. As expected, polymers R1, E44 and inulin promoted growth of *Bacteroides* / *Prevotella* group in faecal cultures. These results may explain the variations and rearrangements detected previously by us in members of these microbial groups by PCR-DGGE when faecal bacteria were incubated in the presence of EPS from intestinal bifidobacteria in a non pH-controlled batch culture (Salazar et al., 2008). Changes in microbial levels as well as in the different species populations of *Bacteroides* / *Prevotella* group may be affecting other microorganisms of the complex colonic ecosystem and the metabolic activities occurring therein (van Tongeren et al., 2005).

Our previous data suggested that the propionic acid production and therefore the acetic / propionic acid ratio could be shifted by the incubation of faecal microorganisms in the presence of EPS (Salazar et al., 2008). In an attempt to correlate shifts in SCFA with changes in faecal

bacteria populations, we used the probe Prop 853 to monitor variations in the *Clostridium* cluster IX since many intestinal propionate-producing bacteria fall within this group of microorganisms (Walker et al., 2005). *Clostridium* cluster IX only became enriched in the presence of the polymer R1 and glucose. Therefore, stimulation of this group of microorganisms may account for the production of propionate when EPS R1 was present. The increase in *Bacteroides*, a group producing variable amounts of propionate depending on the culture conditions (Macfarlane and Gibson, 1997), in the presence of both microbial EPS and inulin, also provides a probable rationale for the increase of propionic acid and decrease of the acetic to propionic acid ratio observed in later cultures (72 h) with these carbohydrates. A reduction in the acetic to propionic acid ratio has been proposed as an indicator of a potential hypolipidemic effect of prebiotics (Delzenne and Kok, 2001). The highest levels of propionic acid and lowest values of the acetic to propionic acid ratio among the four carbohydrates tested were obtained for EPS E44 and the lowest levels of propionic and highest acetic to propionic acid ratio with glucose. These data corroborate our previous findings with the same EPS E44 in non pH-controlled batch cultures (Salazar et al., 2008).

The majority of butyrate-producing bacteria are thought to be included in *Clostridium* clusters XIVa+b and IV. *Roseburia* / *Eubacterium rectale* and *Faecalibacterium* groups, belonging to the first and second clusters respectively, are probably the most prolific butyrate producers in the human colon (Barcenilla et al., 2000; Hold et al., 2003; Pryde et al., 2002). In our study, neither of the EPS tested nor inulin or glucose supported the growth of microorganisms from clusters XIVa+b and IV. The behaviour of these faecal microbial groups was in agreement with the poor butyrate production mediated by EPS but did not provide a rationale for moderately higher levels of this SCFA obtained with inulin. It has been reported that pH values around 6.5 –

7.0 such as those used in the present work can impair butyrate production and promote propionic acid formation by intestinal microbiota (Walker et al., 2005).

In spite of major similarities found between both microbial EPS analysed in our study with respect to their role as fermentable substrates by the colonic microbiota, some differences in their fermentation performances should be highlighted. Thus, polymer R1 but not E44, promoted a slight increase of *Atopobium* and *Clostridium* cluster IX groups during fermentation, suggesting a broader range of potential microbial fermenters for the former carbohydrate. However, the higher levels of SCFA and the lower acetic to propionic acid ratio obtained with E44 with respect to R1 points towards a more efficient utilisation of E44 as carbon source. Whether differences between both substrates are related to their different chemical composition and origin, cannot be clearly concluded from the present data and deserve further attention.

Relating the production of EPS in food and *in vivo* environments, polymer R1 may be synthesized by the producer strain during food fermentation if the strain is included as adjunct culture. On the other hand, although the synthesis of EPS by bifidobacteria in the intestinal environment has not been demonstrated yet, we recently reported the induction *in vitro* of EPS synthesis by bile in *Bifidobacterium* (Ruas-Madiedo et al., 2009). In this way, bile or other stressing factors of the gastrointestinal tract could act as triggering agents of the synthesis of these biopolymers in the gut environment.

In conclusion, this study revealed that relevant changes in the populations of some major anaerobic intestinal bacterial groups as well as noticeable shifts in SCFA and lactic acid production can result from the presence of bifidobacterial EPS. Microbial population dynamics correlated well and supported the SCFA and lactic acid variations detected. EPS could reach the colon by two different ways: they could be either synthesized by probiotics in food products or by

intestinal bacteria in the intestinal environment, thus contributing to the modulation of the intestinal microbiota.

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Table 1. FISH oligonucleotide probes used in this study.

Probe	Target sequence (5'-3')	Target bacterial group/species	Hybridisation T (°C)	References
Erec 482	GCTTCTTAGTCARGTACCG	<i>Clostridium</i> clusters XIVa +b	50	Franks et al. (1998)
Bac 303	CCAATGTGGGGGACCTT	<i>Bacteroides-Prevotella</i> group	46	Manz et al. (1996)
Bif 164	CATCCGGCATTACCACCC	<i>Bifidobacterium</i> genus	50	Langendijk et al. (1995)
Lab158	GGTATTAGCAYCTGTTTCCA	<i>Lactobacillus-Enterococcus</i> group	50	Harmsen et al. (1999)
Ato 291	GGTCGGTCTCTCAACCC	<i>Atopobium</i> cluster	50	Harmsen et al. (2000)
Prop 853	ATTGCGTTAACTCCGGCAC	<i>Clostridium</i> cluster IX	50	Walker et al. (2005)
Chis 150	TTATGCGGTATTAATCTYCCTTT	<i>Clostridium histolyticum</i> group clusters I, II	50	Franks et al. (1998)
Fpra 655	CGCCTACCTCTGCACTAC	Part of <i>Clostridium</i> cluster IV (<i>F. prausnitzii</i> group)	58	Hold et al. (2003)

TABLE 2. Bacterial populations (Log cells/mL) in pH-controlled faecal batch cultures from three donors at 0, 10, 24, 34 and 48 hours using glucose, inulin or EPS as carbon sources. †EPS R1 was isolated from a *Bifidobacterium animalis* strain of dairy origin and EPS E44 was obtained from a human strain of *Bifidobacterium longum*. Total bacterial counts were obtained using DAPI and bacterial groups by FISH probes indicated in Table 1. (*) Significantly different from negative control cultures within the same time point, with the same probe ($P < 0.05$). Substrates with different letters differ depending on the time of incubation, within the same substrate, with the same probe ($P < 0.05$).

Mean $\pm$ SD										
Carbon source	Time (h)	Total Bacteria	<i>Bifidobacterium</i> genus	<i>Bacteroides-Prevotella</i> group	<i>Clostridium</i> cluster XIVa+b	<i>Lactobacillus-Enterococcus</i> group	<i>Atopobium</i> cluster	<i>C.histolyticum</i> group	<i>Clostridium</i> cluster IX	<i>F.prausnitzii</i> group
Control (without carbohydrate added)	0	8.99 $\pm$ 0.10	7.97 $\pm$ 0.10	8.43 $\pm$ 0.27	8.28 $\pm$ 0.07	6.86 $\pm$ 0.14	7.71 $\pm$ 0.26 ^b	7.42 $\pm$ 0.12 ^a	7.56 $\pm$ 0.09	6.84 $\pm$ 0.25
	10	9.22 $\pm$ 0.13	7.82 $\pm$ 0.23	8.54 $\pm$ 0.22	7.95 $\pm$ 0.32	6.47 $\pm$ 0.25	7.69 $\pm$ 0.34 ^b	8.01 $\pm$ 0.15 ^b	7.59 $\pm$ 0.42	7.55 $\pm$ 0.13
	24	9.15 $\pm$ 0.07	7.70 $\pm$ 0.14	8.49 $\pm$ 0.24	7.98 $\pm$ 0.40	6.60 $\pm$ 0.15	7.66 $\pm$ 0.26 ^b	8.05 $\pm$ 0.22 ^b	7.60 $\pm$ 0.49	7.52 $\pm$ 0.44
	34	9.20 $\pm$ 0.08	7.52 $\pm$ 0.32	8.52 $\pm$ 0.11	7.94 $\pm$ 0.34	6.66 $\pm$ 0.04	7.69 $\pm$ 0.27 ^b	8.03 $\pm$ 0.44 ^b	7.77 $\pm$ 0.39	7.20 $\pm$ 0.31
	48	9.13 $\pm$ 0.04	7.53 $\pm$ 0.02	8.21 $\pm$ 0.54	7.92 $\pm$ 0.35	6.65 $\pm$ 0.26	6.83 $\pm$ 0.28 ^a	7.93 $\pm$ 0.11 ^b	7.67 $\pm$ 0.48	7.12 $\pm$ 0.24
EPS-IPLA R1†	0	8.98 $\pm$ 0.13 ^a	8.01 $\pm$ 0.10 ^{ab}	8.45 $\pm$ 0.29	8.26 $\pm$ 0.12	6.84 $\pm$ 0.11	7.64 $\pm$ 0.25 ^{ab}	7.43 $\pm$ 0.09 ^a	7.56 $\pm$ 0.09 ^a	6.95 $\pm$ 0.23
	10	9.22 $\pm$ 0.12 ^b	8.23 $\pm$ 0.23 ^b	8.57 $\pm$ 0.42	8.15 $\pm$ 0.15	7.12 $\pm$ 0.34(*)	7.85 $\pm$ 0.18 ^b	7.88 $\pm$ 0.16 ^{ab}	8.10 $\pm$ 0.18 ^b	7.08 $\pm$ 0.11
	24	9.27 $\pm$ 0.08 ^b	8.29 $\pm$ 0.19 ^b (*)	8.54 $\pm$ 0.34	8.05 $\pm$ 0.30	7.39 $\pm$ 0.31(*)	7.95 $\pm$ 0.10 ^b	7.90 $\pm$ 0.14 ^b	8.19 $\pm$ 0.16 ^b	7.15 $\pm$ 0.30
	34	9.22 $\pm$ 0.11 ^b	8.28 $\pm$ 0.22 ^b (*)	8.74 $\pm$ 0.54	7.86 $\pm$ 0.56	7.61 $\pm$ 0.27(*)	7.37 $\pm$ 0.53 ^{ab}	8.02 $\pm$ 0.18 ^b	8.15 $\pm$ 0.03 ^b	7.02 $\pm$ 0.69
	48	9.17 $\pm$ 0.04 ^b	7.75 $\pm$ 0.14 ^a	8.96 $\pm$ 0.17(*)	7.67 $\pm$ 0.56	7.29 $\pm$ 0.71	6.90 $\pm$ 0.47 ^a	8.13 $\pm$ 0.05 ^b	8.27 $\pm$ 0.16 ^b	7.30 $\pm$ 0.59
EPS-E44 †	0	9.00 $\pm$ 0.13 ^a	8.00 $\pm$ 0.10	8.42 $\pm$ 0.26 ^a	8.27 $\pm$ 0.15	6.84 $\pm$ 0.08	7.70 $\pm$ 0.27	7.44 $\pm$ 0.14 ^a	7.65 $\pm$ 0.06	6.75 $\pm$ 0.26
	10	9.22 $\pm$ 0.11 ^b	8.20 $\pm$ 0.40	8.66 $\pm$ 0.37 ^{ab}	8.22 $\pm$ 0.26	7.24 $\pm$ 0.31(*)	7.83 $\pm$ 0.15	7.97 $\pm$ 0.10 ^b	7.77 $\pm$ 0.21	6.95 $\pm$ 0.21
	24	9.37 $\pm$ 0.07 ^b	8.30 $\pm$ 0.19 (*)	8.91 $\pm$ 0.12 ^b	8.00 $\pm$ 0.33	7.68 $\pm$ 0.33(*)	7.92 $\pm$ 0.08	8.22 $\pm$ 0.25 ^b	8.09 $\pm$ 0.17	7.48 $\pm$ 0.48
	34	9.37 $\pm$ 0.13 ^b	8.28 $\pm$ 0.32 (*)	9.02 $\pm$ 0.06 ^b	7.84 $\pm$ 0.43	7.64 $\pm$ 0.43(*)	7.48 $\pm$ 0.39	8.00 $\pm$ 0.33 ^b	8.15 $\pm$ 0.36	7.33 $\pm$ 0.26
	48	9.09 $\pm$ 0.14 ^a	7.65 $\pm$ 0.23	9.02 $\pm$ 0.16 ^b (*)	7.61 $\pm$ 0.79	7.07 $\pm$ 0.72	7.80 $\pm$ 0.40	8.04 $\pm$ 0.22 ^b	8.11 $\pm$ 0.30	7.30 $\pm$ 0.31
Inulin	0	9.00 $\pm$ 0.12	8.00 $\pm$ 0.10 ^{ab}	8.44 $\pm$ 0.28 ^a	8.25 $\pm$ 0.12	6.84 $\pm$ 0.09 ^a	7.71 $\pm$ 0.26	7.43 $\pm$ 0.16 ^a	7.58 $\pm$ 0.10	6.90 $\pm$ 0.22
	10	9.12 $\pm$ 0.01	8.39 $\pm$ 0.27 ^b	8.63 $\pm$ 0.20 ^{ab}	8.16 $\pm$ 0.22	7.41 $\pm$ 0.24 ^{ab} (*)	7.89 $\pm$ 0.15	7.97 $\pm$ 0.12 ^b	8.01 $\pm$ 0.46	7.35 $\pm$ 0.28
	24	9.28 $\pm$ 0.18	8.35 $\pm$ 0.22 ^b (*)	8.78 $\pm$ 0.21 ^{ab}	8.27 $\pm$ 0.26	7.69 $\pm$ 0.21 ^b (*)	7.81 $\pm$ 0.15	7.95 $\pm$ 0.20 ^b	8.10 $\pm$ 0.20	7.32 $\pm$ 0.68
	34	9.36 $\pm$ 0.27	8.07 $\pm$ 0.24 ^{ab} (*)	8.97 $\pm$ 0.14 ^b	8.22 $\pm$ 0.28	7.78 $\pm$ 0.08 ^b (*)	7.58 $\pm$ 0.12	8.05 $\pm$ 0.16 ^b	8.05 $\pm$ 0.37	7.25 $\pm$ 0.82
	48	9.39 $\pm$ 0.11	7.60 $\pm$ 0.12 ^a	9.00 $\pm$ 0.16 ^b (*)	8.07 $\pm$ 0.39	6.95 $\pm$ 0.64 ^a	7.47 $\pm$ 0.46	8.03 $\pm$ 0.13 ^b	8.04 $\pm$ 0.15	7.18 $\pm$ 0.52
Glucose	0	8.99 $\pm$ 0.10 ^a	7.99 $\pm$ 0.10 ^{ab}	8.40 $\pm$ 0.28	8.28 $\pm$ 0.08	6.82 $\pm$ 0.11 ^a	7.66 $\pm$ 0.24	7.41 $\pm$ 0.12 ^a	7.57 $\pm$ 0.08 ^a	6.89 $\pm$ 0.23
	10	9.29 $\pm$ 0.02 ^b	8.53 $\pm$ 0.36 ^b	8.78 $\pm$ 0.24	7.96 $\pm$ 0.16	7.26 $\pm$ 0.23 ^{ab} (*)	7.76 $\pm$ 0.24	8.14 $\pm$ 0.16 ^b	8.00 $\pm$ 0.26 ^b	6.91 $\pm$ 0.59
	24	9.33 $\pm$ 0.14 ^b	8.43 $\pm$ 0.16 ^b (*)	8.71 $\pm$ 0.09	8.08 $\pm$ 0.15	7.47 $\pm$ 0.14 ^b (*)	7.68 $\pm$ 0.05	8.20 $\pm$ 0.19 ^b	8.10 $\pm$ 0.08 ^b	7.28 $\pm$ 0.64
	34	9.21 $\pm$ 0.11 ^b	8.26 $\pm$ 0.23 ^{ab} (*)	8.68 $\pm$ 0.20	7.99 $\pm$ 0.22	7.56 $\pm$ 0.49 ^b (*)	7.84 $\pm$ 0.10	7.98 $\pm$ 0.21 ^b	8.14 $\pm$ 0.15 ^b	7.12 $\pm$ 0.35
	48	9.13 $\pm$ 0.18 ^a	7.92 $\pm$ 0.09 ^a (*)	8.52 $\pm$ 0.17	8.02 $\pm$ 0.18	6.85 $\pm$ 0.29 ^a	7.20 $\pm$ 0.60	8.04 $\pm$ 0.08 ^b	8.09 $\pm$ 0.20 ^b	7.05 $\pm$ 0.35

Table 3. Concentrations (mM) of lactate and propionate in pH-controlled faecal batch cultures from three donors using glucose, inulin or EPS from bifidobacteria as carbon sources. * EPS R1 was isolated from a *Bifidobacterium animalis* strain of dairy origin and EPS E44 was obtained from a human strain of *Bifidobacterium longum*.

Carbon source	Time (h)	Donor 1		Donor 2		Donor 3	
		Lactate	Propionate	Lactate	Propionate	Lactate	Propionate
Control (without carbohydrate added)	0	0.00	0.51	0.00	0.20	0.94	0.48
	10	1.89	1.27	1.59	1.30	0.00	3.34
	24	0.00	0.79	0.00	2.18	0.00	3.57
	34	0.00	0.65	0.00	8.77	0.00	2.84
	48	0.00	0.89	0.00	3.12	0.00	2.28
	72	0.00	0.58	0.00	3.87	0.00	1.20
EPS IPLA R1 *	0	0.48	0.42	0.58	0.48	1.05	0.63
	10	2.32	0.55	3.30	0.82	2.29	6.76
	24	0.85	5.97	0.75	2.87	0.26	13.52
	34	1.89	7.42	0.65	2.45	0.00	12.74
	48	0.83	8.77	0.00	3.82	0.00	13.05
	72	0.97	11.44	0.00	5.08	0.00	13.62
EPS E44 *	0	1.09	0.41	0.00	0.18	1.14	0.39
	10	9.65	0.56	6.49	0.86	3.63	8.02
	24	14.09	2.87	0.59	4.20	0.33	17.20
	34	15.56	4.06	0.00	7.27	0.00	18.27
	48	16.74	4.82	0.00	8.74	0.00	20.73
	72	5.11	15.65	0.00	10.64	0.00	23.53
Inulin	0	0.34	0.44	0.36	0.46	1.08	0.54
	10	1.16	0.59	2.40	1.29	8.18	5.87
	24	0.38	1.70	3.36	11.09	0.45	11.78
	34	4.30	3.14	1.41	12.16	0.00	11.75
	48	9.54	5.18	0.35	11.98	0.00	12.28
	72	0.00	10.62	0.21	12.94	0.00	13.03
Glucose	0	0.00	0.33	0.00	0.48	0.37	0.32
	10	5.53	1.39	17.33	1.30	21.92	3.42
	24	3.00	4.81	1.34	5.65	12.16	6.67
	34	2.91	4.74	0.00	5.62	9.49	6.40
	48	2.30	5.39	0.00	3.32	5.09	6.59
	72	1.63	5.53	0.00	6.30	0.00	5.93

TABLE 4. Molar concentration of total SCFA (mM) and percent of total SCFA for three major SCFA along with the ratio of acetate to propionate in pH-controlled faecal batch cultures from three donors using glucose, inulin, or EPS as carbon sources. † EPS R1 was isolated from a *Bifidobacterium animalis* strain of dairy origin and EPS E44 was obtained from a human strain of *Bifidobacterium longum*. A, acetate; P, propionate; B, butyrate

Carbon source	Time (h)	Donor 1					Donor 2					Donor 3				
		A	P	B	Ratio A/P	Total SCFAs	A	P	B	Ratio A/P	Total SCFAs	A	P	B	Ratio A/P	Total SCFAs
Control (without carbohydrate added)	0	63.69	36.31	0.00	1.75	1.41	84.84	15.16	0.00	5.60	1.33	77.06	22.94	0.00	3.36	2.08
	10	82.20	17.80	0.00	4.62	7.16	78.17	21.83	0.00	3.58	5.95	78.32	17.58	4.10	4.46	19.00
	24	87.22	12.78	0.00	6.83	6.16	81.28	18.72	0.00	4.34	11.64	85.01	14.99	0.00	5.67	23.84
	34	87.30	9.79	2.91	8.92	6.61	65.43	33.85	0.72	1.93	25.91	80.38	19.62	0.00	4.10	14.46
	48	86.73	13.27	0.00	6.54	6.72	79.34	19.97	0.69	3.97	15.62	47.47	52.53	0.00	0.90	4.34
	72	87.36	12.64	0.00	6.91	4.61	78.21	21.79	1.40	3.59	17.76	51.27	48.73	0.00	1.05	2.46
EPS IPLA R1 †	0	72.30	22.15	5.55	3.26	1.88	76.36	23.64	0.00	3.23	2.03	75.10	24.90	0.00	3.02	2.53
	10	86.67	10.84	2.49	8.00	5.11	88.71	9.63	1.66	9.21	8.47	69.42	28.16	2.41	2.47	24.02
	24	65.64	32.33	2.02	2.03	18.47	82.05	17.34	0.61	4.73	16.52	63.04	31.68	5.28	1.99	42.66
	34	65.58	33.92	0.49	1.93	21.86	85.65	13.60	0.75	6.30	18.00	63.26	32.05	4.69	1.97	39.73
	48	65.39	33.83	0.78	1.93	25.85	79.17	18.80	2.03	4.21	20.33	62.54	31.77	5.69	1.97	41.07
	72	67.17	31.71	1.12	2.12	36.04	44.95	44.36	10.69	1.01	11.46	62.68	30.05	7.26	2.09	45.30
EPS E44 †	0	64.05	27.11	8.84	2.36	1.51	83.02	16.98	0.00	4.89	1.05	90.10	9.90	0.00	9.10	3.91
	10	94.47	4.40	1.13	21.49	12.85	89.82	8.77	1.41	10.24	9.82	73.16	26.84	0.00	2.73	29.88
	24	84.50	13.25	2.25	6.38	21.70	75.70	21.59	2.71	3.51	19.44	59.15	36.91	3.94	1.60	46.60
	34	80.68	16.80	2.51	4.80	24.17	70.19	27.59	2.22	2.54	26.37	57.98	37.77	4.25	1.54	48.39
	48	77.46	17.31	5.23	4.48	27.87	66.13	29.58	4.29	2.24	29.56	55.73	39.51	4.76	1.41	52.48
	72	56.36	39.67	3.97	1.42	39.45	57.89	31.90	10.21	1.81	33.35	54.47	38.96	6.57	1.40	60.39
Inulin	0	70.83	29.17	0.00	2.43	1.50	63.99	25.01	11.00	2.56	1.83	78.45	17.90	3.64	4.38	3.02
	10	72.51	13.51	13.98	5.37	4.34	82.05	12.31	5.64	6.67	10.48	62.52	21.78	15.70	2.87	26.96
	24	70.86	10.91	18.23	6.50	15.60	61.96	26.97	11.07	2.30	41.12	50.70	32.58	16.71	1.56	36.15
	34	58.51	13.99	27.50	4.18	31.48	61.34	27.50	11.15	2.23	44.22	49.76	32.22	18.01	1.54	36.45
	48	59.90	17.22	22.88	3.48	30.05	59.66	26.48	13.86	2.25	45.24	49.56	33.08	17.36	1.50	37.13
	72	50.63	30.36	19.02	1.67	35.00	59.01	26.67	14.32	2.21	48.51	47.17	32.70	20.13	1.44	39.85
Glucose	0	87.56	12.44	0.00	7.04	2.65	62.92	37.08	0.00	1.70	1.28	87.24	12.76	0.00	6.84	2.52
	10	90.56	9.44	0.00	9.60	14.77	93.40	6.60	0.00	14.15	19.66	89.00	10.37	0.63	8.59	32.95
	24	79.36	20.64	0.00	3.84	23.30	67.59	22.05	10.36	3.07	25.62	76.76	16.81	6.43	4.57	39.69
	34	76.87	20.59	2.54	3.73	23.01	66.47	21.67	11.86	3.07	25.91	73.69	16.49	9.82	4.47	38.83
	48	74.91	22.23	2.86	3.37	24.23	72.32	13.84	13.84	5.22	23.98	68.23	17.28	14.49	3.95	38.11
	72	74.13	21.99	3.88	3.37	25.17	64.59	22.41	13.00	2.88	28.12	59.80	16.95	23.25	3.53	34.98

Legends

Figure 1. Chemically simulated gastric and intestinal transit of the EPS fractions isolated from *B. longum* subsp. *longum* IPLA E44 and *B. animalis* subsp. *lactis* IPLA R1 represented as the average amount of EPS determined by SEC before simulation (white bars), after gastric digestion (grey bars) and after intestinal digestion (black bars). Vertical lines indicate standard deviation.

Fig. 1