Title: Exopolysaccharide-producing *Bifidobacterium* strains elicit different *in vitro* response upon human cells

Running title: Bifidobacterial exopolysaccharides interact with human cells

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ABSTRACT

Probiotic foods intended for human nutrition are gaining economic relevance but it is clear that for the application of new strains scientific and rational selection criteria must be used. In this work, the role of exopolysaccharide (EPS) produced by *Bifidobacterium* strains in their potential beneficial effect was studied. Two *in vitro* biological models were used to test the interaction of seventeen strains and their polymers with the host. In general, the EPS-producing bifidobacteria showed good adherence properties to Caco2 and HT29 cell lines which could be of interest for a transitory colonization of the gut. Most purified EPS were able to slightly stimulate the proliferation of peripheral blood mononuclear cells and their cytokine production pattern, depending on the polymer type tested. In general, neutral high molar mass EPS diminished the immune response, whereas acidic and smaller polymers were able to elicit an increased response. Thus, EPS synthesised *in situ* during dairy fermentations could be involved in some of the beneficial effects attributed to the EPS-producing bacteria. Probiotic functionality of these strains seems to be related to the physico-chemical characteristics of EPS as is known to occur with the technological properties.

Keywords: probiotic food; exopolysaccharide; bifidobacteria; intestinal cellular line; PBMC; cytokine

1. Introduction

Nowadays there is increasing evidence that members of the intestinal microbiota are important to maintain the host homeostasis by, among other factors, avoiding the invasion or colonization of pathogens and promoting the host immune response. Cross-talk communication between bacteria – bacteria (inter and intra-species) and bacteria – host (inter-kingdom) take place in this gut scenario by means of different signalling molecules (Curtis, & Sperandio, 2011). Thus the idea to keep a healthy microbial balance, or to restore the microbiota homeostasis in patients suffering from diseases related to dysbiosis through the use of probiotics, is gaining popularity (Gareau, Sherman, & Walker, 2010). Conceptually, probiotics have been defined as “live microorganisms, which when administered in adequate amounts confer a health benefit on the host” (FAO-WHO, 2006). Probiotics for human consumption mostly belong to genera *Lactobacillus* and *Bifidobacterium* and dairy products are the main, but not exclusive, food carrier currently used for their delivery (Margolles, Mayo, & Ruas-Madiedo, 2009; Rivera-Espinoza, & Gallardo-Navarro, 2010). In fact, the choice of the food carrier as well as the food manufacture conditions are of pivotal relevance for probiotic performance since these bacteria, specially bifidobacteria, are very sensitive to technological stresses such as the presence of oxygen (Ebel, Martin, Le, Gervais, & Cachon, 2011; Odamaki, Xiao, Yonezawa, Yaeshima, & Iwatsuki, 2011).

Several surface macromolecules of bacteria, including the previously indicated Gram-positive genera, could act as signal molecules to interact with the host (Lebeer, Vanderleyden, & de Keersmaecker, 2010; Sánchez, Urdaci, & Margolles, 2010). Glycosylated structures from the bacterial surface may interplay with the host cells, and these glycomolecules could be of biological, biotechnological or medical interest (Moran, Holst, Brennan, & von Itzstein, 2009). In this way, exopolysaccharides (EPS) are

glycopolymers present on the surface of many bacteria, including several lactic acid bacteria (LAB) and bifidobacteria, which can either be covalently linked to bacterial surface forming a capsule or loosely attached, or they can be totally secreted in the environment. EPS-producing LAB strains have been traditionally used for the manufacture of fermented dairy products due to the ability of these polymers to confer desirable sensorial attributes, such as an increase in viscosity or firmness and improving stability and texture (Ruas-Madiedo, Salazar, & de los Reyes-Gavilán, 2009). In the context of probiotic foods, the synthesis of EPS could be a selective advantage for probiotic bacteria in order to survive the adverse conditions of the upper part of the gastrointestinal tract after food ingestion (Salazar et al., 2011; Stack, Kearney, Stanton, Fitzgerald, & Ross, 2010). It is not clear whether EPS could favour or avoid the transitory colonization of the gut by the EPS-producing bacteria (Denou et al., 2008) but, we have found that after oral administration of EPS-producing bifidobacteria strains in milk suspensions, the total bifidobacteria population of the intestinal content of rats significantly increased, being able to modify the bacterial population and metabolic activity of the intestinal microbiota (Salazar et al., 2011). A similar effect was previously found with the purified polymers in human faecal fermentations (Salazar et al., 2009b). On the other hand, it has been demonstrated that EPS isolated from *Lactobacillus* and *Bifidobacterium* strains were able to counteract the toxic effect of bacterial toxin and enteropathogens, thus having a potential benefit to the host (Medrano, Hamet, Abraham, & Pérez, 2009; Ruas-Madiedo et al., 2010; Wang, Gänzle, & Schwab, 2010). In addition, other health benefits exerted by probiotics have been attributed to their EPS; this is the case of the immune modulating capability of some of these biopolymers (Nishimura-Uemura et al., 2003; Sato et al., 2004; Yasuda, Serata, & Sako, 2008; Bleau et al., 2010).

On the basis of this background, the aim of this work was to evaluate the role of EPS in the capability of the EPS-producing bifidobacteria to interact with human-host cells, since the production of EPS with beneficial effects could be another criterion for the rational selection of strains aimed for probiotic application in functional foods. For this purpose, seventeen EPS-producing *Bifidobacterium* strains of different origin were used to test their ability to adhere to, and to modify, the proliferation of epithelial intestinal cell lines (Caco2 and HT29). Furthermore, the stimulation response and the cytokine production pattern of peripheral blood mononuclear cells (PBMC) elicited by ten purified EPS-fractions and 3 representative-species (UV-irradiated) of the EPS-producing bacteria were studied as well. Finally, a correlation between the biological function and the physico-chemical characteristics of some EPS polymers was tentatively established.

2. Material and methods

2.1 Bacterial strains and culture conditions

Bifidobacterium strains used in this study are collected in Table 1. Stocks of strains were kept at -80°C in MRSC [MRS (Biokar Diagnostics, Beauvais, France) + 0.25% L-cysteine (Sigma Chemical Co., St. Louis, MO, USA)] containing 20% glycerol. As standard procedure, stocks were grown overnight in 10 ml MRSC cultures at 37°C in the anaerobic chamber MG500 (Don Whitley Scientific, Yorkshire, UK) under 80% (v/v) N₂, 10% CO₂ and 10% H₂ atmosphere. Cultures were used to inoculate (2% v/v) fresh MRSC broth which was incubated for 24 h under the same conditions. These 24 h-grown cultures were used as inocula for the experiments included in this work.

2.2 Exopolysaccharide isolation and purification

EPS-crude fractions from each bifidobacteria were obtained as previously described (Ruas-Madiedo, Gueimonde, Margolles, de los Reyes-Gavilán, & Salminen, 2006). Briefly, 24 h-grown cultures were used to seed the surface of agar-MRS plates which were incubated for 5 days in anaerobic conditions before collecting bacterial biomass using ultrapure water. Then, bacterial suspensions were mixed with 1 volume 2N NaOH and kept overnight at room temperature with mild stirring. After centrifugation to eliminate bacteria, the released EPS was precipitated using 2 volumes of cold absolute ethanol for 2 days at 4°C. The precipitated EPS was dissolved in ultrapure water and dialysed using 12-14 kDa MWCO dialysis tubes (Sigma) against ultrapure water for 3 days at 4°C, with daily changes of water. Finally, the dialyzed sample was freeze-dried to obtain the EPS-crude fraction. A second purification procedure, conducted in order to reduce the DNA and protein content, was carried out. To this end, each EPS-crude fraction was dissolved (5 mg/ml) in buffer solution (50 mM Tris-HCl, 100 mM MgSO₄·7H₂O, pH 7.5) and DNase I from bovine pancreas (Sigma) was added (final concentration 2.5 µg/ml), followed by incubation for 6 h at 37°C. Afterwards, pronase E from *Streptomyces griseus* (Sigma) dissolved in buffer (50 mM Tris-HCl, 2% EDTA, pH 7.5) was added (final concentration 50 µg/ml) and the solution was incubated for 18 h at 37°C. Then proteins and peptides were precipitated by adding TCA (final concentration 12%) keeping the mixture under stirring for 30 min at room temperature. After centrifugation (6000 xg, 20 min, 4°C), the pH of supernatant was increased to 4.5 – 5.0 with 10N NaOH, intensively dialysed under conditions previously described and finally freeze-dried. This corresponds with the EPS-purified fraction that was used for further experiments. The protein content of each EPS fraction was determined using the BCA protein assay (Pierce, Rockford, IL, USA) following the manufacturer's instructions. The yield and protein content of both EPS-crude and EPS-purified fractions are shown in the supplementary material (table S1).

2.3 Interaction of EPS-producing bifidobacteria with epithelial intestinal cell lines

2.3.1 Cell lines and culturing conditions

Two epithelial intestinal cell lines were used in this study: Caco2 (ECACC 86010202) and HT29 (ECACC 91072201) purchased from the European Collection of Cell Cultures (Salisbury, UK). Both cell lines express characteristics of differentiated enterocytes upon reaching confluence; however, among other different traits, they present variable ranges of permeability since Caco2 is composed of solely absorptive cells whereas HT29 presents mucus-producing Goblet cells (Hilgendorf et al. 2000). The culturing and maintaining conditions of the cell lines were achieved using standard procedures (Sánchez, Fernández-García, Margolles, de los Reyes-Gavilán, & Ruas-Madiedo, 2010), and the media and supplements used are collected in the supplementary material (table S2). For adhesion experiments each cell line was seeded (around 1×10^5 cells/ml) in 24-well plates BD-Falcon (BD Biosciences, San Diego, CA, USA) and incubated at 37°C, 5% CO₂ atmosphere in a CO₂-Series Shel-Lab incubator (Sheldon Manufacturing Inc., OR, USA) and used in a confluent-differentiated stay (13 ± 1 days). For proliferation assays, cells were seeded in the same numbers in fluorometry-validated 96-well plates Optilux™ BD-Falcon (BD Biosciences) and incubated in the same conditions until 3 ± 1 days. In all cases, cell lines were washed twice with Dulbecco's PBS (Sigma) to remove the antibiotics of media before the start of each experiment.

2.3.2 Bacterial adhesion to cell lines

The capability of the seventeen EPS-producing bifidobacteria strains to adhere to the two cell lines was tested and compared with that of *B. animalis* subsp. *lactis* Bb12, used as the reference strain. Bacterial cultures were washed twice with Dulbecco's PBS and resuspended in the corresponding culture medium of each cell line without antibiotics.

Bacteria and confluent- cell monolayers were co-cultured (ratio 10: 1) for 1 h in a HERAcell® 240 incubator (Thermo Electron LED GmbH, Langenselbold, Germany) at 37°C, 5% CO₂. Afterwards, monolayers were washed twice with Dulbecco's PBS, to remove the non-adhered bacteria, and trypsinised with trypsin-EDTA (Sigma) to release the eukaryotic cells. For bifidobacteria counts (CFU/ml), serial dilutions in Ringer's solution (Merck, Darmstadt, Germany) were made, pour-plated in agar-MRSC and incubated for 3 days at 37°C in anaerobic conditions. Adhesion percentage was calculated from the ratio of bacteria adhered/ bacteria added. Strains were tested in quadruplicate: two replicated cell-monolayer plates, each of them using two wells per strain.

To evaluate the effect of purified EPS on bacterial adhesion, additional assays were carried out with the cell line HT29 and strains *B. longum* NB667, *B. longum* 667Co and *B. animalis* subsp. *lactis* Bb12 in the presence / absence of the purified EPS-fractions NB667 and 667Co. In this case, bacterial suspensions of each strain were prepared in three MacCoy's medium (MM) without antibiotics: MM (control), MM supplemented with 1 mg/ml EPS-NB667 and MM supplemented with 1 mg/ml EPS-667Co. Adhesion assays were carried out as previously described.

2.3.3 Proliferation of cell lines in presence of bacteria

The ability of the EPS-producing bifidobacteria strains to modify the proliferation of the two cell lines was assessed by means of the immunoenzymatic BrdU Proliferation Assay (Calbiochem, Merck). Cell lines in proliferative state (3±1 days) were inoculated with bacterial suspensions prepared as previously described (ratio 1: 10, respectively) and co-cultivated for 24 h in the HERAcell-240 incubator. In the control sample, each cell line was kept with its corresponding medium without bacterial addition. Six hours after inoculation the BrdU label was added and then incubation was continued. Afterwards, cells were fixed and the proliferation assay was carried out according to the manufacturer's

instructions. The fluorescence emitted (420 nm) after sample excitation (325 nm) was recorded in a Cary Eclipse fluorescence spectrophotometer (Varian Ibérica, S.A., Madrid, Spain). The proliferation index (PI) was calculated as follows: (fluorescence emitted by the sample/ fluorescence emitted by the control) -1. Measurements were carried out in quadruplicate: duplicated wells in each of the two replicated microplates.

2.4 Interaction of EPS with human peripheral blood mononuclear cells (PBMC)

2.4.1 Isolation of PBMC

Human PBMC were obtained from standard buffy-coat preparations from 9 routine blood donors (Asturias Blood Transfusion Center, Oviedo, Spain) by centrifugation over Ficoll-Hypaque gradient (Lymphoprep, Nycomed, Oslo, Norway). All blood donors were healthy adult volunteers without any pathology or treatment. Approval for this study was obtained from the Regional Ethics Committee for Clinical Investigation (Asturias, Spain). Medium and supplements used for PBMC cultivation are collected in supplementary material (table S2).

2.4.2 Proliferation of PBMC and cytokine production

The proliferation of PBMC in the presence of ten EPS-purified fractions was determined by quantifying [³H]-thymidine incorporation in cultured cells. For this, 2×10⁴ PBMC were seeded in 96-well round-bottom microtiter plates (Costar, Cambridge, MA, USA) in 200 µL of RPMI-1640 medium containing 1 µg/ml of each EPS-purified fraction. Parallel cultures were made without stimuli (reference control) or were treated with 50 ng/ml LPS from *E. coli* 0111:B4 (Sigma) as a positive control. After 4 days of incubation at 37°C, 5% CO₂, 75 µl supernatant from these cultures were collected and stored at -20°C until use for cytokine determination. Afterwards, the same amount of RPMI medium containing 1 µCi [³H]-thymidine per well was re-added. After 16 h of additional

incubation, cells were harvested onto glass fiber filters and the cell-incorporated [³H]-thymidine counts were performed using a standard scintillation technique (Packard Instruments, Downers Grove, IL, USA). Results were expressed as a stimulation index (SI), which was calculated as the ratio between the counts per minute (cpm) measured in stimulated samples and the cpm values measured in the reference control. Measurements were made in triplicate wells for each of the 9 donor cultures.

Additionally, the proliferation of PBMC in the presence of UV-irradiated (non-viable) bacteria was determined for one representative strain of each species: *B. animalis* A1dOxR, *B. longum* E44 and *B. pseudocatenulatum* C52, following the procedure described above. UV-irradiated bacteria were prepared as previously described (López, Gueimonde, Margolles, & Suárez, 2010) and co-cultivated with PBMC in ratio 5:1. Samples for cytokine analysis were also collected.

Cytokine levels in PBMC culture supernatants were quantified by a multiplex immunoassay “cytometric bead array” (CBA) using the Becton Dickinson FACS Canto II flow cytometer (BD Biosciences) and the FCAP array software (BD Biosciences). The CBA Flex Set (BD Bioscience) includes IFN γ , TNF α , IL-12, IL-8, IL-10, IL-1 β and IL-17 cytokines and assay conditions were those recommended by the manufacturer. The detection limits were: 0.8 pg/ml IFN γ , 3.7 pg/ml TNF α , 1.9 pg/ml IL-12p70, 3.6 pg/ml IL-8, 3.3 pg/ml IL-10, 7.2 pg/ml IL-1 β and 0.3 pg/ml IL-17.

2.5. Statistical analyses

The SPSS/PC 15.0 software package (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. Data of bacterial adhesion to cell lines, as well as proliferation of cell lines and PBMC, followed normal distribution, thus independent one-way ANOVAs were used for analyses. Data of cytokine production by PBMC were not normally

distributed; therefore, the non-parametric Mann-Whitney test for 2-independent samples was used. The comparisons performed for each set of data are described in the corresponding figure or table legend.

3. Results and Discussion

3.1. Interaction of EPS-producing bifidobacteria with intestinal cells

Accordingly to one of the criteria recommended by FAO/WHO for selection of putative probiotic strains, the adhesion capability of seventeen EPS-producing bifidobacteria strains to Caco2 and HT29 epithelial cell lines (Figure 1) as well as the influence of EPS polymers in this property (Table 2) were studied. In general, for a given strain the adhesion capability was similar in both cell lines although some strains (e.g. A1dOx and E44) showed different behaviour, which could be related with the differential intrinsic characteristic of both cell lines as mentioned above. Half of our strains showed higher ($p < 0.05$) adhesion percentage than the reference *B. animalis* subsp. *lactis* Bb12, which is considered a good adherent strain (He et al., 2001). As has been previously reported, the adhesion capability was not associated with species but it was a characteristic of strain (Blum et al., 1999; Laparra & Sanz, 2009). In fact, 4 out of our 5 *B. animalis* strains showed similar or lower adhesion than the reference strain Bb12 belonging to the same species. Furthermore, strains very closely related among them, such as the triad *B. animalis* A1, A1dOx and A1dOxR and the pair *B. longum* NB667 and 667Co, showed differences in adhesion. The parental strains (A1 and NB667) were less adhesive ($p < 0.05$, statistical analysis not shown) than their corresponding bile-salt resistant derivatives, obtained by adaptation of parental strains to increasing concentrations of bovine bile salt or sodium cholate (Noriega, Gueimonde, Sánchez, Margolles, & de los Reyes-Gavilán, 2004). A similar trend was previously detected in these strains when their adhesion was tested

against human intestinal mucus (Gueimonde, Noriega, Margolles, de los Reyes-Gavilán, & Salminen, 2005), thereby indicating that the acquisition of bile salt resistance could have modified the surface properties of the adapted strains. In fact, it is known that in *B. animalis* bile adaptation affected the cellular morphology (Margolles, García, Sánchez, Gueimonde, & de los Reyes-Gavilán, 2003), as well as the bacterial fatty acid composition and membrane fluidity (Ruiz, Sánchez, Ruas-Madiedo, de los Reyes-Gavilán, & Margolles, 2007). But other extracellular structures, such as EPS, could be modified as well. In this way, the molar mass distribution and monosaccharide ratio was different in the EPS fractions synthesized by strains A1, A1dOx and A1dOxR (also named IPLA-R1, Ruas-Madiedo et al., 2010) and high variability was detected as well in the EPS purified from strains of human origin (Salazar et al. 2009a). Therefore, the differential physico-chemical properties of these EPS could account for the variation in the adhesion ability of their producing strains.

To further check the effect of purified EPS on bacterial adhesion, the epithelial intestinal cell line HT29, the EPS NB667 and 667Co and two closely related strains but showing the highest differences in adhesion (*B. longum* NB667 and 667Co), as well as the reference *B. animalis* Bb12 (Table 2), have been chosen. The adhesion of strains Bb12 and 667Co was not modified by any EPS, but the adhesion of strain NB667 increased significantly ($p < 0.05$) in the presence of both polymers. *B. longum* NB667 was the strain presenting the poorest adhesion ability to HT29 cell line among bacteria tested in the present work, its adherence being improved by adding external EPS. It is not clear how EPS are involved in the adhesion of bacteria to intestinal mucosa. Denou and co-workers (2008) showed that a *Lactobacillus johnsonii* mutant strain, lacking EPS biosynthesis, was able to reside for more time in the gut of mice. Thus, in this situation it seems that EPS produced by the parental *L. johnsonii* strain could hinder its adhesion *in vivo* by physically

shielding some bacterial adhesins and thus blocking its interaction with the eukaryotic receptor (Schembri, Dalsgaard, & Klem, 2004). Similarly, our group have previously found that the presence of certain purified-EPS decreased the adhesion *in vitro* of probiotics to human intestinal mucus (Ruas-Madiedo et al., 2006). Thus, it was postulated that this fact may be related to a competitive exclusion between the purified EPS and mucus for binding probiotics. It is worth mentioning that mucus is formed by polymerization of glycoproteins (mucins) which carry oligosaccharides that may act as ligands for microbial adhesins (McGuckin, Lindén, Sutton, & Florin, 2011). Given that bacterial EPS are also polymers of monosaccharides, they could mimic eukaryotic ligands and thereby compete for adhesion of probiotics. From these studies, it is clear that bacterial adhesion to intestinal mucosa is a property highly dependent on the surface characteristic of each strain and extracellular molecules such as EPS could play a role in this process. Since the physico-chemical composition of our polymers is different, it can be hypothesised that these intrinsic EPS properties could be key factors determining their biological effect, as it has been clearly demonstrated for the technological functionality of EPS synthesised by LAB (Ruas-Madiedo et al., 2009).

Some authors have suggested an anticarcinogenic activity of bifidobacterial EPS by *in vitro* testing the effect upon proliferation of different epithelial intestinal cellular lines (Ku, You, & Ji, 2009; You, Oh, & Ji, 2004). However, this single measurement is not suitable to assess such activity, since cancer development is a complex process that involves several cellular events. Nevertheless, the proliferation of intestinal cellular lines could be used as an indicator of the interaction between bacteria and host. In this way, the proliferation of Caco2 and HT29 in the presence of our EPS-producing bifidobacteria and the reference strain Bb12 (Figure 2) was tested. In general, most bifidobacterial strains (12 out of 18) significantly ($p < 0.05$) reduced the proliferation index (PI) of HT29, when

compared with the cell line without bacteria added (control sample) which could be due to a decrease in the rate of DNA synthesis or due to a cellular death (cytotoxicity). However,, a different response to strains was detected in Caco2. Only 5 strains showed the same behaviour (statistically significant) in both cell lines; that is *B. longum* NB667 and *B. pseudocatenulatum* C52, E515 and H34, which decreased the PI, and *B. animalis* A1dOxR which increased this index. However, *B. pseudocatenulatum* A102 showed an opposite tendency in HT29 and Caco2. Thus, results obtained were highly dependent on the cell line tested which underlines the fact that the exclusive measurement of cellular line proliferation is not a valid method to determine the anti-carcinogenic potential of probiotic strains (Grimoud et al., 2010). Several authors have suggested that the immune system of an animal model was very much involved in preventing, or reducing, the tumor proliferation by bacterial EPS. Using animals with pre-implanted tumoral cells (Sarcoma-180), it was reported that EPS produced by LAB had in fact an *in vivo* antitumoral activity (Kitazawa et al., 1991; Oda, Hasegawa, Komatsu, Kambe, & Tsuchiya, 1983); but, these results were not reproducible *in vitro* with cultures of S-180 cells (Kitazawa et al., 1991). Therefore, it seems that the putative antitumoral effect of EPS is likely due to their ability to act as effector molecules to induce response of the host immune system.

3.2 Response of PBMC to EPS and UV-irradiated EPS-producing bacteria

In the current work, the capability of 10 bifidobacterial EPS-purified fractions (1 µg/ml) and 3 UV-irradiated EPS-producing bacteria (ratio bacteria: PBMC 5: 1) to stimulate the proliferation of PBMC obtained from nine healthy donors was evaluated after 4 days of co-incubation (Figure 3). In general, all EPS-purified fractions at concentration 1 µg/ml were able to stimulate the proliferation of the human mononuclear cells, although only significant differences ($p<0.05$) were detected for polymers A1 and NB667, with respect to cells in the absence of stimuli. It is worth noting that the presence of the whole-

inactivated (by UV-radiation) EPS-producing bifidobacteria (bacteria: PBMC ratio 5:1), did not increase the stimulation index of PBMC with respect to their corresponding EPS-purified fractions ($p>0.05$). This suggests, among other possibilities, that the polymers surrounding the bacterial surface probably hinder other potential immune effector molecules. We have previously found that UV-irradiated bifidobacteria are, in general, low inducers of PBMC proliferation with the exception of the strains *B. animalis* IPLA4549 and 4549dOx (López et al., 2010), which do not constitutively produce EPS. Recently, Wu and co-workers (2010) have shown that the proliferation of the macrophage cell line J77A was significantly increased by the EPS (5 µg/ml) produced by *B. longum* BCRC 14634, as well as by the heat-inactivated strain (bacteria: cell line ratio 25:1). Other bifidobacterial-EPS showed a mitogenic effect upon different immune cell cultures, such as the polysaccharide from *B. adolescentis* M101-4 which was able to *in vitro* stimulate the proliferation of murine splenocytes and Peyer's patch cells (Hosono et al., 1997). On the contrary, none of the bifidobacterial-EPS purified from strains isolated from infant faeces were able to stimulate the lymphocyte proliferation of Balb/C mice spleen cultures (Amrouche, Boutin, Prioult, & Fliss, 2006). Our results together with those reported in literature suggest that the physico-chemical characteristics of the EPS molecules are also responsible for their capability to induce proliferation of different immune cells. In this way, Kitazawa and co-workers (1998) have demonstrated that the phosphate present in the acidic EPS synthesized by *Lb. delbrueckii* subsp. *bulgaricus* OLL1073R-1 was the molecule triggering the mitogenic activity of murine splenocytes and Peyer's patch cells. In our case, the EPS A1 also contains phosphate in its chemical composition which was not present in the EPS NB667 (Ruas-Madiedo et al., 2010). In addition, it has been shown that β -glucans from fungus *Sclerotium*, having the same monomer composition but differing in size (molar mass) and spatial conformation, elicited variable proliferation in human

monocyte cultures (Falch, Espevik, Ryan, & Stokke, 2000). Variations in these physico-chemical parameters could also be a possible explanation for differences detected between the closely related EPS A1 and A1dOxR, since the first one lacks the high molecular mass fraction present in the A1doxR polymer (Ruas-Madiedo et al., 2010).

The cytokine production patterns measured in the supernatants collected from the co-cultures PBMC with EPS-purified fractions and PBMC with UV-irradiated bifidobacteria are depicted in Figure 4. In general, no statistical differences were detected for levels of IL-1 β , IL-17 and IL-8 in presence of EPS-purified polymers or EPS-producing bacteria with respect to the control (RPMI medium). The IL-10 concentration was not modified in the presence of EPS but was increased by the EPS-producing bacteria, whereas the IL-12 values did not change, or were significantly lower, when certain EPS were added. Remarkably, all EPS and their corresponding bacteria significantly increased the production of IFN- γ and TNF- α in the stimulated PBMC, with the exception of EPS-A1dOxR.

Taking into account that cytokines are molecules mutually regulated, the balance between pivotal cytokines can influence a microenvironment prone to induce CD4⁺ T-cell differentiation towards Th1, Th2 or Th17 cells. IL-12 is a key cytokine playing a central role in promoting Th1-mediated responses (Dalod et al., 2002). The anti-inflammatory cytokine IL-10 exerts an opposite immunoregulatory effect, suppressing the production of IL-12 and Th1 cytokines (De Smedt et al., 1997). Thus, IL-10 and IL-12 play opposite roles in inflammatory responses, and therefore their relative balance is of central relevance for controlling immune deviation. In addition, recent studies have provided evidence that IL-1 β , in the absence of IL-12 and IL-4, can drive Th17 generation (Annunziato, Cosmi, Liotta, Maggi, & Romagnani, 2008). Taking into account all these data, the observed differences in the production of IL-12, IL-10, TNF- α and IL-1 β between our different EPS-purified fractions or UV-irradiated bifidobacteria could contribute to understanding the type of Th response that they may promote. Thus, several ratios

between cytokines that are relevant for T cell differentiation: Th1 (IL-12/IL-10), Th2 (IL-10/TNF- α) and Th17 (IL-1 β /IL-12) were calculated to predict subsequent possible Th-cell deviation (Table 3). The increased immune response induced by the 3 UV-inactivated EPS-producing bacteria (differences respect to the control, $p < 0.01$) was noticeable, the highest being that elicited by *B. longum* E44. Interestingly, this response was not detected with the corresponding EPS-purified fractions. In addition, differences for the ratio TNF- α /IL-10 between EPS-purified and EPS-producing bacteria were detected for the A1dOxR and E44 pairs. However, the same ratio was obtained for EPS C52 and its *B. pseudocatenulatum* producing strain, which could denote an implication of the EPS C52 in the Th1 response induced by the producing strain. Finally, it is worth noting that no statistically significant differences were detected in the PBMC cytokine pattern stimulated with the EPS-purified fractions from the closely related strains NB667 and 667Co. Similarly, in the case of the triad A1 - A1dOx - A1dOxR, only the EPS A1dOxR induced lower levels of most cytokines, that of TNF- α being significantly lower ($p < 0.05$) with respect to the other two EPS-related fractions. Since, as previously indicated, the EPS A1dOxR has a different chemical composition and bigger size than EPS A1 and A1dOx (Ruas-Madiedo et al., 2010), this result may indicate that the physico-chemical parameters of these polymers could be directly correlated with the immune response that they were able to elicit. Generalizing, the EPS A1dOxR, which is the polymer having the highest molar mass fraction, reduced *in vitro* the immune response. Bleau and co-workers (2010) have recently shown that the high molar mass EPS-fraction obtained from *Lactobacillus rhamnosus* RW-9595M was able to decrease the production of inflammatory cytokines by macrophages. Furthermore, it has been indicated that the high molar mass EPS synthesised by *Lactobacillus casei* Shirota is able to reduce the excessive immune reaction induced by this strain during the activation of macrophages (Yasuda et al., 2008). In addition to the size,

the chemical composition of EPS can also play a role. The acidic (due to the presence of phosphate in its composition) EPS fraction isolated from a strain of *Lb. delbrueckii* subsp. *bulgaricus* elicited immune response in murine macrophages, whereas the neutral EPS fraction did not (Nishimura-Uemura et al., 2003). The introduction of phosphate groups in molecules of dextran synthesised by *Leuconostoc mesenteroides* promoted an increase in the production of IFN- γ and IL-10 on murine splenocytes, thus being able to act as immune stimulators (Sato et al., 2004). Therefore, it has been proposed that some bifidobacterial- and LAB-EPS might be useful as mild immune modulators for macrophages (Makino et al., 2006; Yasuda et al., 2008; Wu et al., 2010), this property being very much dependent on the EPS type (Amrouche et al., 2006).

4. Conclusion

An overall picture from our work indicates that EPS produced by bifidobacteria were able to interact with the host at intestinal epithelia and at immune systemic levels. Certain EPS-producing bifidobacteria, and some of their purified polymers, were able to *in vitro* increase their adhesion to intestinal cell lines. This feature might lead to longer and tighter interactions between bacteria and intestinal mucosa, thus making feasible their contact with GALT (gut associated lymphoid tissue) cells. At systemic level, results of proliferation and cytokine production pattern induced in PBMC by our EPS-purified suggests, first, that results obtained were dependent on the type of EPS and, second, that the EPS-producing strains were able to induce higher *in vitro* immune response than their corresponding EPS-purified fractions, except EPS C52. Thereby, and accordingly with some findings reported in literature, our results strongly suggest that the chemical composition and physical characteristics of bifidobacterial-EPS are key parameters determining the ability of certain polymers to interact with the intestinal epithelial cells or

to act as immune effector molecules. In general, it seems that neutral bigger-size EPS were able to suppress the production of pro-inflammatory cytokines, whereas smaller size EPS, or acidic EPS, had immune stimulating properties. Further research is needed in order to determine which of the intrinsic characteristics of our bifidobacterial-EPS are responsible for the cross-talk with the human host cells. This will help to gain insight to, and understanding of, the mechanisms of immune modulation by bifidobacterial-EPS, to further undertake new strategies to balance the immune homeostasis status associated with dysbiosis or inflammatory disorders.

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645

Table 1 Origin and identification of the *Bifidobacterium* strains used in this work

Species	Strain ¹	Origin	GenBank 16S accession no.	Reference
<i>B. animalis</i>	C64MR*	Rectal mucosa	EU430030	Salazar et al. (2008)
	E43	Human faeces	EU430031	Salazar et al. (2008)
	A1*	Dairy product	-	Ruas-Madiedo et al. (2010)
	A1dOx*	Bile-adapted	-	-
	A1dOxR*	Bile-adapted	GU586289	Salazar et al. (2011)
	Bb12 ²	Collection	CP001853	Garrigues et al. (2010)
<i>B. longum</i>	IPLA20001*	Breast milk	HM856586	Arbolea et al. (2011)
	E44*	Human faeces	EU430035	Salazar et al. (2008)
	H67	Human faeces	EU430032	Salazar et al. (2008)
	H73	Human faeces	EU430033	Salazar et al. (2008)
	L55	Human faeces	EU430034	Salazar et al. (2008)
	NB667 ^{3,*}	Collection	-	Noriega et al. (2004)
	667Co*	Bile-adapted	-	Noriega et al. (2004)
<i>B. pseudocatenulatum</i>	A102	Human faeces	EU430027	Salazar et al. (2008)
	C52*	Human faeces	EU430029	Salazar et al. (2008)
	E63	Human faeces	EU430025	Salazar et al. (2008)
	E515	Human faeces	EU430026	Salazar et al. (2008)
	H34G*	Human faeces	EU430028	Salazar et al. (2008)

¹ Asterisk indicates that the EPS-purified fraction isolated from these strains was used in the PBMC proliferation and cytokine production assays.

² Culture collection of Christian Hansen (Hørsholm, Denmark)

³ Culture collection of NIZO food research (Ede, The Netherlands)

Table 2 One-way ANVOVA analysis of the adhesion to the cell line HT29 of strains *B. animalis* Bb12, *B. longum* NB667 and *B. longum* 667Co performed in the absence and presence of 1 mg/ml of the EPS NB667 and 667Co. Means that within the same column do not share a common superscript letter are statistically different ($p < 0.05$) according to the mean comparison LSD (least significant difference) test.

Culture media ¹	% Adhesion (mean $\pm$ SD)		
	<i>B. animalis</i> Bb12	<i>B. longum</i> NB667	<i>B. longum</i> 667Co
MM (control)	0.38 $\pm$ 0.14	0.13 $\pm$ 0.04 ^a	0.72 $\pm$ 0.18
MM+EPS-NB667	0.36 $\pm$ 0.14	0.25 $\pm$ 0.09 ^b	0.70 $\pm$ 0.15
MM+EPS-667Co	0.27 $\pm$ 0.09	0.25 $\pm$ 0.06 ^b	0.76 $\pm$ 0.11
		*	

¹ MM: McCoy's Medium

Table 3 Ratios of cytokines produced by PBMC co-cultivated with 10 purified EPS (1 µg/ml) synthesised by *Bifidobacterium* species or with 3 UV-irradiated EPS-producing bifidobacteria (ratio 5:1) for 4 days. Statistical analysis was assessed by means of the non-parametric Mann-Whitney test for 2-independent samples. Differences with respect to the control medium (RPMI) are represented by asterisks (** p<0.01). Additionally, comparisons between “purified EPS” and “EPS-producing bacteria” were carried out for 3 pairs of data, using the same test, and values that do not share equal superscript letter are different (p<0.05).

Species	Stimuli	Mean ± SD (pg/ml)		
		TNFα/ IL-10	IL-10/ IL-12	IL-1β/ IL-12
<i>B. animalis</i>	RPMI	0.99±1.56	178.31±295.98	3092.09±2999.51
	LPS <i>E. coli</i>	2.83±4.26	190.34±157.73	4347.24±2886.70
	C64	6.16±13.17	90.65±119.95	2764.41±3217.20
	A1	2.13±2.89	66.55±71.29	1783.24±1666.98
	A1dOx	1.84±2.08	98.07±78.28	4360.76±3174.89
	A1dOxR-EPS A1dOxR-strain	1.34±1.46 ^a 6.76±6.25 ^{b**}	34.33±40.03 ^a 940.34±1473.62 ^b	3170.98±3498.34 ^a 3454.50±3643.36 ^a
<i>B. pseudocatenulatum</i>	H34	1.46±1.54	145.16±110.93	3340.11±3026.55
	C52-EPS	5.64±8.83 ^a	121.81±110.52 ^a	3318.15±2975.51 ^a
	C52-strain	5.81±5.04 ^{a**}	1065.91±1693.72 ^a	3495.56±3497.07 ^a
<i>B. longum</i>	NB667	1.46±1.31	24.58±37.56	1989.07±4108.08
	667Co	2.39±2.37	66.04±79.69	2468.90±3868.62
	IPLA20001	2.49±3.38	52.99±34.37	930.97±949.07
	E44-EPS	3.62±4.09 ^a	157.91±151.04 ^a	2156.40±1743.51 ^a
	E44-strain	17.92±11.95 ^{b**}	922.76±1473.13 ^a	4692.77±3584.73 ^a

Figure legends

Fig. 1. Percentage of adhesion (CFU bacteria adhered / CFU bacteria added) of 17 EPS-producing bifidobacteria to the epithelial intestinal cellular lines Caco2 and HT29. Data were compared with values obtained for the reference strain *B. animalis* subsp. *lactis* Bb12 (dotted line) by means of independent one-way ANOVAs and differences ($p < 0.05$) are marked with an asterisk.

Fig. 2. Proliferation index (PI) of the epithelial intestinal cell lines Caco2 and HT29 in the presence of 17 EPS-producing bifidobacteria (grey bars) and the reference strain *B. animalis* subsp. *lactis* Bb12 (black bar). Proliferation was determined in the control sample, which corresponds to each cell line growing in their specific culture medium without bacteria added. The PI was calculated as: (fluorescence emitted by sample / fluorescence emitted by the control) -1. Statistical differences were analysed with respect to the control by means of independent one-way ANOVA tests, and they are annotated with an asterisk.

Fig. 3. Stimulation index (SI) of peripheral blood mononuclear cells (PBMC) co-cultured for 4 days with different stimuli: 10 purified-EPS (1 $\mu\text{g/ml}$) synthesized by different bifidobacteria (light-grey bars), LPS (50 ng/ml) from *E. coli* (black bar) and 3 UV-irradiated EPS-producing bacteria (ratio bacteria: PBMC 5: 1, dark-grey bars). Stimulation was measured as well in the control sample, which corresponds with the PBMC growing RPMI medium without stimuli added. The SI was calculated as $[\text{}^3\text{H}]\text{-thymidine cpm}$ measured in stimulated samples / cpm measured in the control sample. Statistical differences were analyzed with respect to the control (dotted line) by means of independent one-way ANOVA tests, and they are marked with an asterisk.

696

697 **Fig. 4.** Cytokine profile produced by peripheral blood mononuclear cells (PBMC) co-
698 cultured for 4 days in the presence of 10 purified-EPS (1 μ g/ml) synthesized by different
699 bifidobacteria and LPS (50 ng/ml) from *E. coli*, as well as by 3 UV-irradiated whole
700 bifidobacteria (ratio bacteria: PBMC 5:1). The control sample corresponds to PBMC
701 growing in RPMI medium. For each cytokine (pg/ml), the “box and whiskers” figure
702 represents median, interquartile range and minimum and maximum values, calculated from
703 cultures of PBMC isolated from nine healthy donors. The non-parametric Mann-Whitney
704 test for 2-independent samples was used to assess differences between each sample and the
705 control sample, which are indicated with an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).
706 Additionally, the same test was used to determine differences between each pair purified-
707 EPS and EPS-producing bacteria. In this case, samples that do not share the same letter
708 within each pair are statistically different ($p < 0.05$).

709

710 **Figure 1**

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712 **Figure 2**

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714 **Figure 3**

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