Title: Characterisation of the exopolysaccharide (EPS)-producing *Lactobacillus paraplantarum* BGCG11 and its non-EPS producing derivative strains as potential probiotics

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Running headline: Probiotic potential of *Lactobacillus paraplantarum*

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ABSTRACT

Traditional fermented foods are the best source for the isolation of strains with specific traits to act as functional starters and to keep the biodiversity of the culture collections. Besides, these strains could be used in the formulation of foods claimed to promote health benefits, i.e. those containing probiotic microorganisms. For the rational selection of strains acting as probiotics, several *in vitro* tests have been proposed. In the current study, we have characterised the probiotic potential of the strain *Lactobacillus paraplantarum* BGCG11, isolated from a Serbian soft, white, homemade cheese, which is able to produce a "ropy" exopolysaccharide (EPS). Three novobiocin derivative strains, which have lost the ropy phenotype, were characterised as well in order to determine the putative role of the EPS in the probiotic potential. Under chemically gastrointestinal conditions, all strains were able to survive around 1-2% (10^6 - 10^7 cfu/ml cultivable bacteria) only when they were included in a food matrix (1% skimmed milk). The strains were more resistant to acid conditions than to bile salts and gastric or pancreatic enzymes, which could be due to a pre-adaptation of the parental strain to acidic conditions in the cheese habitat. The ropy EPS did not improve the survival of the producing strain. On the contrary, the presence of an EPS layer surrounding the strain BGCG11 hindered its adhesion to the three epithelial intestinal cell lines tested, since the adhesion of the three non-ropy derivatives was higher than the parental one and also than that of the reference strain *Lactobacillus rhamnosus* GG. Aiming to propose a potential target application of these strains as probiotics, the cytokine production of peripheral blood mononuclear cells (PBMC) was analysed. The EPS-producing *L. paraplantarum* BGCG11 strain showed an anti-inflammatory or immunosuppressor profile whereas the non-ropy derivative strains induced higher pro-inflammatory response. In addition, when PBMC were stimulated with increasing concentrations of the purified ropy EPS (1, 10 and 100 μg/ml) the cytokine profile was similar to that obtained with the EPS-producing lactobacilli, therefore pointing to a putative role of this biopolymer in its immune response.

Keywords: *Lactobacillus paraplantarum*, exopolysaccharide, probiotic, intestinal cellular line, PBMC, cytokine.

1. Introduction

Lactic acid bacteria (LAB) have been used for food fermentations since ancient times and some of them, due to their long history of safe use in human consumption, currently have the "Qualified Presumption of Safety" (QPS) status (EFSA, 2007). Nowadays functional cultures, which "contribute to food safety and/or offer one or more organoleptic, technological and nutritional, or health advantages", have been widely implemented in the manufacture of fermented foods (Leroy and De Vuyst, 2004). In addition to the synthesis of lactic acid, there are several LAB traits that could confer desirable attributes to specific fermented products; this is the case of exopolysaccharide (EPS) production in dairy fermentations. Bacterial EPS are a large group of carbohydrate polymers which could be either covalently associated with the cell surface forming a capsule, or be loosely attached, or totally secreted into the environment of the cell (Ruas-Madiedo et al., 2008). The *in situ* synthesis of EPS by LAB cultures during milk fermentation improves the viscosity and texture of the fermented product since these biopolymers act as thickeners and emulsifiers or could also be fat replacers in low-caloric products. Besides, it seems that the synthesis of EPS could help the producing bacteria to survive adverse environmental conditions and it has been proposed that these polymers could promote some benefit for human health (Ruas-Madiedo et al., 2008).

Apart from foods, several LAB genera such as *Lactobacillus* are naturally inhabitants of the gastrointestinal tract (GIT) of animals including the human gut (Margolles et al., 2009) where EPS-producing strains have also been isolated (Ruas-Madiedo et al., 2007; Tieking et al., 2005). The presence in this ecological niche of members of this genus is one of the reasons for which some lactobacilli species have been proposed as beneficial for human health. According to the definition proposed in 2001 by FAO/WHO expert consultation group, probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2006). The concept of probiotic is

linked to strain level and it cannot be extended to species. Therefore, studying probiotic traits of new or commercial strains is of special relevance before their application in the formulation of functional foods (Jensen et al., 2012). For this purpose, a guideline has been proposed by FAO/WHO (2006) for testing the probiotic potential of a given strain based on several safety and health criteria which can be achieved by means of *in vitro* and/or *in vivo* approaches. Nevertheless, the efficacy of a probiotic formulation should be demonstrated by means of well designed double-blind, placebo controlled human clinical trials (FAO/WHO, 2006).

The EPS-producing strain *Lactobacillus paraplantarum* BGCG11 (formerly classified as *Lactobacillus casei* CG11) was isolated from soft, white, homemade cheese and showed a "ropy" phenotype, denoted by the formation of a long filament in the culture, which is due to the synthesis of EPS (Kojic et al., 1992). The biopolymer is a heteropolysaccharide mainly composed of glucose (76%) and rhamnose (21%) with some traces of galactose (Cerning et al., 1994). Preliminary studies revealed that genetic information for production of this EPS is possibly located in the 30 kb plasmid, since several novobiocin-cured (Muc^-) derivative strains lacking this plasmid have lost their ropy character and were not able to produce significant amounts of EPS in comparison to the parental (Muc+) strain (Cerning et al., 1994; Kojic et al., 1992). The aim of the current work was to *in vitro* evaluate the EPS-producing *L. paraplantarum* BGCG11 strain and its derivatives as potential probiotics and to determine the putative role that the EPS could play in this feature. For this purpose, bacterial suspensions in milk of the parental BGCG11 and three Muc^- derivative strains were challenged to the chemically simulated GIT transit. Their ability to adhere to different epithelial intestinal cell lines and their capability to modify the proliferation and cytokine production by peripheral blood mononuclear cells (PBMC) were analysed as well.

2. Material and Methods

2.1. Bacterial growth and strain identification

The EPS-producing ropy *L. paraplantarum* BGCG11 parental strain and its non-ropy (Muc$^-$) derivatives obtained after treatment with novobiocin (strains NB1, NB4, NB16) were cultured in MRS broth (Merck, Darmstadt, Germany) at 30°C. Total DNA from these cultures was extracted and quantified using standard procedures. The determination of the species identity was performed by nucleotide sequencing using primers complementary to 16s rDNA: UNI16SF and UNI16SR (Jovcic et al., 2009). The discrimination among *Lactobacillus plantarum*, *L. paraplantarum* and *Lactobacillus pentosus* was carried out by multiplex PCR *recA* gene-derived primers plantF, pentF, and plantR as described (Torriani et al., 2001). All PCR amplifications were performed using the KAPA *Taq* DNA polymerase kit (Kapa Biosystems, MA, USA). Reaction mixtures contained: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 50 mM each of the dNTPs, 1 U of *Taq* polymerase, 5 pM of each primer (for multiplex PCR 0.25 µM of each primer), and 0.1 µg of template DNA in a final volume of 50 µl. The PCR products were purified with QIAquick PCR Purification KIT (Qiagen, Hilden, Germany) and were sequenced by Macrogen (Seoul, Korea). The BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST) was used to determine the most related DNA sequences in the NCBI GenBank database. The nucleotide sequence of 16S rDNA of the strain *L. paraplantarum* BGCG11 is held in the "European Nucleotide Archive" database (http://www.ebi.ac.uk/ena) with the accession number HE600693. Additionally, the strain was typed by AFLP method in the Laboratory for Microbiology, Ghent University (Ghent, Belgium).

2.2. EPS extraction and purification

For EPS extraction and purification, the ropy parental strain BGCG11 was grown in basal minimum media (BMM) containing amino acids, minerals, and vitamins (Cerning et al., 1994) and supplemented with 10 g/l saccharide-free Casamino Acids (Difco, Michigan, USA) and with 20 g/l glucose (see supplementary Table S1). Fresh medium was inoculated (10%, v/v) with overnight BMM-grown culture and incubated at 25 °C for 48 h.

After incubation, bacteria were removed by centrifugation (12,000 x *g*, 30 min, 4 °C) and EPS were extracted and precipitated at 4 °C for 48 h by adding 2 volumes of chilled absolute ethanol. The precipitate was collected by centrifugation (12,000 x *g*, 20 min, 4 °C), dissolved in distilled water and dialysed against water, using 12-14 kDa MWCO membranes (Sigma Chemical Co., St. Louis, MO, USA), for 24 h at 4 °C. The dialysed-retentate was freeze-dried to obtain the EPS-crude fraction which was additionally purified to reduce the DNA and protein content. For this purpose, the EPS-crude powder was dissolved in 50 mM Tris-HCl, 10 mM $MgSO_4 \cdot 7H_2O$ (pH 7.5) at a final concentration of 5 mg/ml and treated with DNAse type-I (Sigma, final concentration 2.5 µg/m) for 6 h at 37 ºC, followed by Pronase E (Sigma, final concentration 50 µg/ml) treatment for 18 h at 37 ºC. Afterwards, TCA (12% final concentration) was added, to precipitate enzymes and residual amino acids or peptides, and the mixture was stirred at room temperature for 30 min. After centrifugation (12,000 x *g*, 20 min, 4 ºC), the supernatant was collected, its pH adjusted to 4.0-5.0 with 10 M NaOH, dialysed against ultra-pure water (for 3 days, at 4 ºC with a daily change of water) and finally freeze-dried. The resulting lyophilised powder was the purified EPS-CG11 fraction, which was used in different concentrations for further analyses. The protein content of this EPS-fraction was quantified by the BCA method (Pierce, Rockford, IL, USA), the DNA presence was determined using the Take3™ Multi-volume Plate (Biotek Instruments GmbH, Bad Friedchshall, Germany) and the EPS content was determined by means of SEC-MALLS analysis, as indicated in the next section.

2.3. Simulated GIT transit of L. paraplantarum *strains and the purified EPS-CG11*

The survival of *L. paraplantarum* BGCG11 parental strain and its Muc$^-$ derivatives after the chemically simulated GIT transit was determined as described by Sánchez et al. (2010). In short, 24 h MRS-grown cultures were harvested, washed twice with 0.85% NaCl and concentrated 10-times in reconstituted (10%) sterilised skimmed-milk (BD, Difco®, Becton Dickinson, Franklin Lakes, NJ, USA). Afterwards, bacterial suspensions were diluted

10-times with gastric juice [GJ: 125 mM NaCl, 7 mM KCl, 45 mM $NaHCO_3$, 0.3% Pepsine (Sigma) adjusted to pH 2 with HCl], incubated for 90 min at 37 ºC in aerobic conditions under stirring (≈ 200 rpm). Then, bacterial suspensions were centrifuged (2,050 x*g*, 15 min), resuspended in duodenal juice [DJ: 1% bovine bile (Sigma) adjusted with 10 M NaOH to pH 8.0] and incubated for 10 min at 37 ºC in anaerobic chamber MG500 (Don Whitley Scientific, West Yorkshire, UK) under 10% (v/v) H_2, 10% CO_2 and 80% N_2 atmosphere. Finally, harvested cell suspensions were resuspended in intestinal juice [IJ: 0.3% bovine bile, 0.1% Pancreas acetone powder porcine Type I (Sigma), pH 8.0] and incubated for 120 min at 37 ºC in anaerobic conditions. Two samples were collected during IJ challenge, after 60 min and 120 min. Determination of viable counts was carried out in the initial cultures and after each of the GJ, DJ and IJ challenges. Serial dilutions of the samples were made in Ringer´s solution (Merck) and pour-plated into agar-MRS. Plates were incubated for 48 h at 30 ºC and results were expressed as cfu/ml. The percentage of survival was calculated from the viable counts recovered after each chemically simulated GIT step with respect to the initial counts (% cfu recovered bacteria / cfu initial bacteria). Experiments were carried out in triplicate.

The simulated transit through GIT of isolated EPS was performed according to Salazar et al. (2009b). Briefly, the EPS-purified fraction was dissolved in ultra-pure water (Sigma) at 10 mg/ml and submitted to independent GJ and DJ stresses. In both cases, 100 µl of EPS solution was mixed with either 900 µl of GJ or DJ and incubated under conditions previously described for both challenges. Afterwards, samples were collected and stored at -80 ºC before EPS-degradation analysis. In the case of the GJ samples, the pH was increased until 4.5 ± 0.5 with 10 M NaOH before storage. The hydrolysis of the EPS-purified fractions was assessed by means of size exclusion chromatography (SEC) coupled with a multi-angle laser light scattering (MALLS) detector. The chromatographic system (Waters, Milford, MA, USA) was composed of an Alliance 2690 module injector, a Photodiode Array PDA 996 detector (checked at 220 and 280 nm for protein detection or 260 nm for DNA detection), a 410

refractive index (RI) detector and the Empower software (Waters). The RI detector allowed us to determine the amount of EPS present in the sample using calibration curves made with dextran standards (Salazar et al, 2009a). Additionally, the MALLS detector (Dawn Heleos II, Wyatt Europe GmbH, Dembach, Germany) was coupled in series and the software Astra 3.5 was used for molar mass distribution analysis. Separation 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) at 40 ºC, flow rate 0.45 ml/min using 0.1 M $NaNO_3$ as mobile phase. Experiments were repeated three times.

2.4. Adhesion of L. paraplantarum *strains to intestinal cell lines*

The colonocyte-like cell lines Caco2, HT29 and HT29-MTX were used to determine the adhesion ability of the parental and the three Muc- *L. paraplantarum* derivative strains, using *Lactobacillus rhamnosus* LMG18243 (also named GG) as reference strain. *L. paraplantarum* strains were grown in MRS broth, under conditions previously described, and strain GG was grown in MRS supplemented with 0.05% L-cysteine (Sigma) at 37 ºC, 5% CO_2 in a Heracell® 240 incubator (Thermo Electron LDD GmbH, Langenselbold, Germany).

Caco2 and HT29 were purchased from the European Collection of Cell Cultures (ECACC No. 86010202 and 91072201, respectively) and HT29-MTX was kindly supplied by Dr. T. Lesuffleur (Lesuffleur et al., 1990). The culture and maintenance of the cell lines were carried out following standard procedures (Sánchez et al., 2010) using DMEM medium for Caco2 and HT29-MTX, and McCoy´s medium for HT29, supplemented with foetal bovine serum and with a mixture of antibiotics (50 μg/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamicin and 1.25 μg/ml amphotericin B). Media and reagents were purchased from Sigma. Intestinal cells were seeded in 24-well plates and cultivated until a confluent differentiated state was reached. For adhesion experiments, 13±1 day-old cellular monolayers were used.

Lactobacilli were cultured for 24 h and after washing twice with Dulbecco´s PBS solution (Sigma) were resuspended in the corresponding cell-line media without antibiotics at

a concentration of about 10^8 cfu/ml. Cellular monolayers were also carefully washed and bacterial suspensions were added at a ratio of about 10:1 (bacteria: eukaryotic cell). Adhesion experiments were carried out for 1 h at 37 °C, 5% CO_2 and, afterwards, wells were gently washed to release unattached bacteria before proceeding with the lysis of cellular monolayers using 0.25% Trypsin–EDTA solution (Sigma). Dilutions of samples, before and after adhesion, were made in Ringer´s solution and bacterial counts were performed in agar-MRS. The adhesion was calculated as: % cfu adhered bacteria /cfu added bacteria. Experiments were carried out in two replicated plates and in each plate two wells were used per sample.

2.5. Proliferation and cytokine production of PBMC in the presence of non-viable **L. paraplantarum** *strains or the EPS-CG11 purified fraction*

Human PBMC were obtained from buffy-coats from routine blood donors (Asturian Blood Transfusion Center, Oviedo, Spain) after the approval of the Regional Ethics Committee for Clinical Investigation (Asturias, Spain). PBMC were isolated by centrifugation over Histopaque-1077 gradient (Sigma) of buffy-coats from 6 healthy donors. PBMC were cultured in RPMI-1640 medium, containing 2 mM L-glutamine, 25 mM Hepes (PAA, Pasching, Austria), 10% (v/v) heat-inactivated foetal bovine serum (PAA) and supplemented with streptomycin and ampicillin (100 μg/ml).

The PBMC response to the four UV-irradiated lactobacilli, as well as to different concentrations (1, 10 and 100 μg/ml) of the EPS-CG11, was determined according to López et al. (2010) as follows: 2×10^4 PBMC suspended in 200 μl of RPMI medium were co-incubated with bacteria at a ratio of 1:5 (PBMC: bacteria) or with EPS for 4 days at 37 ºC, 5% CO_2. PBMC cultured in complete RPMI media and in the presence of 0.05 μg/ml lipopolysaccharide (LPS) from *E. coli* 0111:B4 (Sigma) were used as negative and positive proliferation controls, respectively. Each stimulus and control was tested in triplicate wells per PBMC donor, using 96-well round-bottom microtiter plates (Costar, Cambridge, MA, USA). After 4 days of incubation, 20 μl supernatants of each replicate-well were collected,

mixed and stored at -20ºC before being used for cytokine analysis. The same volume of complete RPMI medium containing 10 μM (final concentration) BrdU label (GE Healthcare Ltd., Buckinghamshire, UK) was added and incubation was continued for 18 h. After this time, eukaryotic cells were collected by centrifugation, fixed and their proliferation was determined using the Amersham Cell Proliferation Biotrack ELISA system (GE Healthcare Ltd.) following the manufacturer´s instructions. The colorimetric reaction was measured at 450 nm in a Modulus Microplate photometer (Turner Biosystems, CA, USA) and the proliferation index (PI) obtained for each stimulus was referred to the negative control (that is, the proliferation index of the control is 1).

The cytokine production from PBMC co-cultures was quantified by means of the multiplex immunoassay Flex Set for CBA (Cytometric Bead Array) including IFNγ, TNFα, IL-12, IL-8, IL-10, IL-1β and IL-17 (Becton Dickinson, BD Biosciences, San Diego, CA). The FacsCantoII flow cytometer and the FCAP array software (BD Biosciences) were used for analysis. The detection limits were: 0.8 pg/ml (IFNγ), 3.7 pg/ml (TNFα), 1.9 pg/ml (IL-12p70), 3.6 pg/ml (IL-8), 3.3 pg/ml (IL-10), 7.2 pg/ml (IL-1β), and 0.3 pg/ml (IL-17).

2.6. Statistical analysis

The SPSS 15.0 software for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Data of chemically simulated GIT transit, bacterial adhesion to epithelial cell lines and PBMC proliferation were normally distributed and therefore they were analysed by means of independent one-way ANOVA tests. When necessary, the mean comparison test LSD (least significant difference, $p < 0.05$) was used. The cytokine production by PBMC was analysed by the non-parametric Mann-Witney test for two independent samples comparing values obtained for each stimulus with respect to that of the negative control (PBMC cultured in RPMI).

3. Results

3.1. Identification of L. paraplantarum strains

The strain BGCG11 was previously classified as *L. casei* (Kojic et al., 1992). However, the results obtained in this study revealed that the 16S rDNA sequence of BGCG11strain showed 99% identity with sequences of *L. plantarum* and *L. pentosus*. The multiplex PCR with *recA* gene-derived primers, which allows differentiation among *L. plantarum*, *L. pentosus* and *L. paraplantarum* species, was performed and an amplicon of 107 bp corresponding with that expected for *L. paraplantarum* was obtained (data not shown). Additionally, the AFLP fingerprinting showed that the resulting profile corresponded to that of this species. These results enabled the re-classification of the parental strain BGCG11 and its Muc- derivatives NB1, NB4 and NB16 strains as *L. paraplantarum* species.

3.2. In vitro *GIT survival and intestinal adhesion of* L. paraplantarum *strains*

The ropy EPS-producing *L. paraplantarum* BGCG11 and its non-ropy derivative strains tested in this study were able to survive GIT challenges only when bacteria were suspended in skimmed milk (Figure 1). They did not survive when bacterial suspensions were made in buffered solution (data not shown). The survival percentage of bacteria in milk suspensions to gastric digestion was around 80% for all strains tested, which indicates that *L. paraplantarum* was resistant to acidic conditions. In addition, a short exposure time to high bile salts concentrations (1%) decreased the survival up to 70%. However, prolonged exposure times to lower bile concentrations (0.3%) and pancreatic enzymes drastically reduced the bacterial viability. The final survival rate varied between 1 and 2% (10^6 to 10^7 cfu/ml), the highest ($p<0.05$) being that of the derivative NB1 strain (initial Log cfu/ml: parental BGCGG11 8.5 ±0.1, derivatives NB1 9.1±0.01, NB4 8.9±0.1 and NB16 8.9±0.1).

The protein content of the initial lyophilised EPS-CG11 was 1.97±0.17 % and no DNA was detected using either the Take3™-Multi-volume Plate or the PDA detector (260 nm) of the HPLC. Using data from the SEC-MALLS analysis, it has been estimated that the polymer content was 98.25±1.02% and it was found that the EPS-CG11 presented two peaks of different size (see supplementary Figure S1); the highest peak had a molar mass about

285 $2.2x10^6$ Da (peak abundance 89.3%) and the smallest about $5.6x10^4$ Da (abundance 10.7%).
286 The presence of more than one fraction of different size was previously reported in different
287 EPS synthesised by strains of food origin (Sánchez et al., 2005; Vaningelgem et al., 2004) and
288 human origin (Salazar et al., 2009a). In order to check whether GIT challenges have modified
289 the physico-chemical characteristics of the EPS-CG11 was submitted the native polymer to
290 gastric or intestinal digestion. The SEC-MALLS analysis of challenged polymer showed that
291 the amount of the highest-peak EPS-CG1, as well as its molar mass, remained without
292 statistical noticeable modifications (Table 1). This indicates that EPS-CG11 was stable under
293 simulated GIT digestion.

294 The adhesion ability of parental strain BGCG11 and its derivatives to the epithelial
295 intestinal cell lines Caco2, HT29 and HT29-MTX was determined in order to denote possible
296 differences related with the production of a ropy-EPS by the BGCG11 strain. *L. rhamnosus*
297 GG was used as the positive reference strain, since it has well recognised adherence to
298 intestinal mucosa and is a strain widely included in many probiotic foods. In general, the
299 adhesion properties of the five strains tested followed the same tendency in the three intestinal
300 cell lines, although lower percentages of adhesion were detected for the mucus-producing
301 HT29-MTX cell line (Figure 2). Probably, the presence of the glycoprotein (mucin) layer in
302 this cellular line hinders the availability of cells receptors for bacteria. The non-ropy
303 derivatives *L. paraplantarum* NB1, NB4 and NB16 were able to adhere significantly ($p<0.05$)
304 better than the reference strain GG in all cell lines, having adhesion percentages ranging from
305 5% to 25%. On the contrary, the parental *L. paraplantarum* BGCG11 showed similar (HT29
306 and HT29-MTX) or lower (Caco2, $p<0.05$) adhesion ability than *L. rhamnosus* GG (which
307 was around 3%). Therefore, these results showed that the non-ropy derivatives had a better
308 adherence trait than the parental strain and the reference strain GG, pointing to a possible
309 involvement of the EPS interfering with the interaction with epithelial intestinal cells.

310 ***3.3.* In vitro *response of PBMC elicited* by L. paraplantarum *strains and EPS-CG11***

311 The capability of the *L. paraplantarum* BGCG11 and its derivatives, inactivated by
312 UV-radiation, as well as the purified EPS-CG11 at three concentrations (1, 10 and 100 μg/ml)
313 to elicit immune response was tested upon PBMC isolated from six healthy human donors.
314 The proliferation indexes of PMBC measured in the presence of these stimuli and LPS (0.05
315 μg/ml) from *E. coli*, as positive control, showed that all bacterial strains were able to
316 significantly ($p<0.05$) increase the proliferation of PBMC in comparison with the control
317 (RPMI in the absence of stimulus, Table 2). On the contrary, neither the LPS from *E. coli* nor
318 the EPS-CG11 at any concentration modified the proliferation of PBMC.
319 The cytokine production pattern of PBMC co-cultured for 4 days with the different
320 previously described stimuli shows that, in general, the four *L. paraplantarum* strains showed
321 significantly higher ($p<0.05$) levels of all cytokines tested than the negative control sample
322 (RPMI without stimulus) except for the IL-17 production (Figure 3). The comparison of each
323 strain with respect to the control showed that the parental EPS-producing strain BGCG11 did
324 not modify the IL-12 levels, whereas the derivative strains significantly increased the levels of
325 this cytokine. Statistical comparisons among the four strains, showed significantly ($p<0.05$)
326 lower levels of TNF-α and IL-12 only for the parental strain, whereas no significant
327 differences were observed among BGCG11 and its derivatives for the rest of analyzed
328 cytokines. The response of PBMC stimulated with different concentrations of the purified
329 EPS-CG11 showed lower production of all cytokines, except IL-17, in comparison with that
330 produced by the lactobacilli strains. Thus, this polymer induced a lower immune response
331 than the whole bacterium. However, an EPS-dose effect was detected since most cytokines
332 increased levels in the presence of higher concentrations of EPS. Specifically, we observed a
333 significant increase of the IL-10 and IL-1β production ($p<0.05$) at the highest (100 μg/ml)
334 EPS concentration tested compared with the negative (RPMI medium) control.
335 Aiming to propose specific target applications, the type of T-helper (Th) response that
336 the four *L. paraplantarum* strains or the EPS-CG11 could induce was evaluated; for this,

different ratios between cytokines that are relevant for T cell differentiation were calculated: TNF-α/IL-10 (pro-Th1, i.e. inflammatory response), IL-10/IL-12 (pro-Th2 -Treg, i.e. anti-inflammatory or suppressor response) and IL-1β/IL-12 (pro-Th17, i.e. relevant for immune mucosa defence), as in healthy individuals, these T cell subsets are adequately balanced. From these observed ratios it seems that, although no statistical differences were detected among strains ($p>0.05$), the parental EPS-producing strain BGCG11 presented the highest IL-10/IL-12 ratio (pro-Th2-Treg) as well as an increased IL-1β/IL-12 proportion (pro-Th17 response), whereas the non-ropy derivatives showed a pro-Th1 profile (Table 3). In fact, the parental strain was the only one that presented a significantly ($p<0.05$) higher Th2-Treg ratio compared with the negative control. Besides, it is worth mentioning that the immune response elicited upon PBMC by purified EPS followed the same pattern as that of the EPS-producing strain, although the differences with respect to the control were statistically significant ($p<0.05$) only at the highest EPS dose (100 μg/ml).

4. Discussion

According to the FAO/WHO guideline for the evaluation of probiotics for human food applications (FAO/WHO, 2006), the bacterial survival of gastric and intestinal digestion is one of the most desirable properties that strains with probiotic potential should present. The performance of the ropy *L. paraplantarum* BGCG11 parental strain, isolated from a home-made cheese, and its non-ropy derivatives challenged against the adverse conditions of the GIT, was adequate taking into account that from a initial 8 Log cfu/ml units, a final 6 Log units still remained in cultivable conditions (around 1-2% survival). The highest survival rate was that of the derivative NB1 strain, which could be due to the slightly higher initial number of cells challenged in this strain in comparison with the other three. In addition, it is important to note that these survival rates were only achieved when bacteria were included in a food matrix (1% skimmed milk). It has previously been reported that the delivery of bacteria in

363 food carriers favours survival through the GIT due to the buffering and protective effect of the
364 food components (Ranadheera et al., 2010).This fact underlines the relevance of the food
365 carrier selection for each specific strain which could as well be related with the original
366 source of bacteria. Our *L. paraplantarum* were resistant to low pH conditions, which could be
367 due to a pre-adaptation to acid conditions in the natural environment from which the parental
368 strain was isolated (Terzic-Vidojevic et al., 2007). Similar behaviour to GIT challenge was
369 reported for other food-origin lactobacilli strains, such as *Lactobacillus delbrueckii* subsp.
370 *lactis* (Burns et al., 2011). Against this, the survival of our *L. paraplantarum* strains quickly
371 declined in the presence of bile salts and pancreatic enzymes, stress factors that isolates of
372 food-origin do not encounter in their natural environment. This could lead to the conclusion
373 that food-origin strains could be less adaptable to the human gut environment, although from
374 previous reports this correlation is not clear (Gaudana et al., 2010; Lee et al., 2011). Indeed, it
375 seems that independent of the ecological niche from which the strains were isolated, both acid
376 and bile resistance are species- and/or strain-dependent traits (Lo Curto et al., 2011; Mathara
377 et al., 2008). Regarding the putative role of EPS in the protection of the producing strain
378 during GIT transit, it seems that the polymer itself was not digested during the challenges.
379 This has been reported for other purified EPS synthesised by LAB and bifidobacteria, which
380 are stable to harsh conditions probably due to their intrinsic resistance to acid hydrolysis
381 (Salazar el al., 2009b). However, the polymer cover present on the surface of the EPS-
382 producing strain *L. paraplantarum* BGCG11 was not effective enough to increase its survival
383 in comparison with the non-ropy derivatives, as also occurred for the EPS-producing strains
384 *L. delbrueckii* subsp. *lactis* 193 and 193+ (Burns et al., 2011). Similarly, the EPS envelope of
385 strains *Bifidobacterium longum* NB667 and 667Co did not improve their survival when *in*
386 *vitro* challenged to human-origin gastric and duodenal juices. In contrast, the EPS layer
387 surrounding several *Bifidobacterium animalis* subsp. *lactis* strains was effective in keeping
388 the number and viability of bacterial cells after simulated GIT transit (de los Reyes-Gavilán et

al., 2011). Thus, the capability of EPS surrounding the producing-bacteria to deal with the harsh conditions of the upper-part of the gut tract seems to be both polymer and strain dependent. In this regard, it has been already indicated that the EPS physico-chemical characteristics, intrinsic to each polymer type, must account for their biological, technological and protective abilities (Ruas-Madiedo et al., 2008). Additionally, EPS production has been proposed as one of the mechanisms that bacteria, including lactobacilli, trigger in presence of GIT challenges (Burns et al., 2010).

Another criterion proposed by FAO/WHO guideline for the selection of strains with probiotic potential is their ability to transiently colonise the intestinal mucosa. Several *in vitro* and *in vivo* models have been used to this aim and, through several of these approaches, it has been shown that the strain *L. rhamnosus* GG has good adherence to intestinal mucosa (Laparra and Sanz, 2009; Vesterlund et al., 2005). Thus, we have used GG strain as the reference to compare the performance of our *L. paraplantarum* strains regarding their ability to adhere to three intestinal epithelial cell lines. These cell lines express morphological and functional differentiated characteristics resembling that of mature enterocytes (Jumarie and Malo, 1991; Lesuffleur et al., 1990). Results obtained with this model showed that our non-ropy *L. paraplantarum* derivatives NB1, NB4 and NB16 had a remarkably better adhesion trait than that of the ropy parental BGCG11 strain or that of the *L. rhamnosus* GG reference strain. In this way, it has also been detected that an isogenic EPS-mutant (strain CMPG5351) of *L. rhamnosus* GG, which has lost the ability to produce big-size galactose-rich EPS and produces only small-size glucose-rich cell-wall associated polysaccharides, had increased adherence to mucus and Caco-2 monolayer cells in comparison to the parental GG (Lebeer et al., 2009). Similarly, a mutant of *Lactobacillus johnsonii* NCC533, with deleted EPS biosynthesis genes, persisted longer in the murine gut presumably because of enhanced exposure of its adhesins (Denou et al., 2008). However, Lebeer et al. (2011) using the same EPS-mutant from *L. rhamnosus* GG have found lower persistence of the EPS-mutant strain

than the parental GG strain in an *in vivo* murine model, which contradicted the previous *in vitro* findings of the same authors. It seems that *in vivo*, the EPS synthesized by strain GG form a protective shield against innate antimicrobial molecules synthesized by the host cells. Therefore, the role of bacterial EPS in the colonization of intestinal mucosa is still not clear. It seems that in some cases the EPS envelope covering the producing strains could hinder bacterial adhesion to enterocytes, whereas in its absence other surface molecules could have been exposed and therefore acted as adhesins. Nevertheless, EPS could also be involved in persistence of producing bacteria by acting as protective molecules against antimicrobial molecules. Besides, and taking into account the citations reported above, the model used to test the ability of any potential probiotic strain to transiently colonize the GIT is of pivotal relevance.

Nowadays, it is well known that probiotics impact on the immune function of humans by inducing T-cell differentiation to Th1, Th2, Th17 or Treg (Dong et al., 2010; López et al., 2011). Therefore, it is claimed that they promote health benefits throughout the regulation of the immune response, However, it is necessary to underline that the immune modulation ability is strictly species- and strain-dependent (Díaz-Ropero et al., 2007; van Hemert et al., 2010), that for a given strain it is dose-dependent (Evrard et al., 2011) and that well documented probiotics are not effective for all human populations (Seifert et al., 2011). In the current study, we have demonstrated that the closely related parental *L. paraplantarum* BGCG11 strain and its novobiocin-cured NB1, NB4 and NB16 elicited different immune response patterns upon human PBMC. The parental BGCG11 strain had increased cytokine ratios suggestive of Th2-Treg and Th17 response, the first involved in suppressive and immunoregulatory functions and the last one being of special relevance for the immune mucosa homeostasis. Although, both T-cell populations seem to be implicated in antagonistic functions, it is known that Th17 and Treg cells share common cytokine signaling pathways allowing plasticity in their polarization. Thus, Treg cells are able to produce IL-17 and

assume a Th17 function when they are activated in the presence of pro-inflammatory cytokines, such as IL-1β (Yang et al., 2008). Interestingly, these data are in agreement with previous results of our laboratory supporting such Treg/Th17 plasticity induced by the strain *Bifidobacterium bifidum* LMG13195 (López et al., 2011). In the current study, and conversely to the parental BGCG11 strain, we have detected that the non-ropy *L. paraplantarum* derivatives induced a pro-Th1 cytokine pattern. Thus it seems that the parental and derivative strains showed an opposite immune response tendency. The main difference among these strains is the production of a ropy, big-size EPS in the parental one, which suggests that the EPS produced by *L. paraplantarum* BGCG11 could play a role in the suppression of the immune response induced by the parental strain. Thus, we hypothesize that the EPS-CG11 could act as an effector molecule regulating the differential immune response. In fact, this seemed to be corroborated when PBMC were co-incubated with different concentrations of the purified EPS-CG11. An EPS dose-effect was detected, showing a similar immune profile to that of the *L. paraplantarum* EPS-producing strain. The physico-chemical characteristics of EPS-CG11 revealed that it is a big-size polymer (around $2x10^6$ Da) and previous work showed that it was mainly composed of glucose (75.7%) and rhamnose (20.5%), with traces of galactose (2.1%), and having a neutral character (Cerning et al., 1994). Thus, it seems that the EPS-CG11 surrounding the producing bacteria down-regulated the immune response, whereas in the non-ropy derivatives the absence of this polymer layer could expose other surface molecules which triggered a type Th1 (pro-inflammatory) response. Similar results have recently been obtained with EPS differing in size produced by closely related *B. animalis* subsp. *lactis* strains as indicated by López et al. (2012). Indeed, these authors suggest that EPS of high size have the ability to act as immune suppressor molecules, whereas small polymers are able to elicit an increased response.

5. Conclusion

Taking all these results together, it could be concluded that the EPS-producing *L. paraplantarum* BGCG11 and its novobiocin derivatives NB1, NB4 and NB16 had different *in vitro* biological and functional properties, probably due to the presence of the EPS layer surrounding the parental strain. Although the *in vitro* and *in vivo* results cannot be directly extrapolated the stains tested in this study could have specific probiotic applications for human consumption. Taking into account the immune parameters, the parental BGCG11 strain, with anti-inflammatory or immunosuppressor profile, could be included in the diet of patients suffering diseases associated with an increased inflammatory status, such as allergy and inflammatory bowel disease or other auto-immune disorders, as well as in functional foods formulated for the elder population. However, following the FAO/WHO criteria and EFSA recommendations, is necessary to underline that the safety and health promoting efficacy of such as functional foods need to be proven in human clinical trials.

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Table 1. Stability of the highest peak of EPS-CG11 (see supplementary Figure S1) submitted to different chemically simulated gastrointestinal tract (GIT) conditions. No statistical differences among challenges were obtained after one-way ANOVA and by the LSD mean comparison tests.

	GIT challenge	**Mean ±SD**
Amount (μg)*	Initial	261.5±2.9
	Gastric juice	285.5±7.8
	Intestinal juice	255.6±7.9
Molar Mass (x10^6 Da)	Initial	2.2±0.6
	Gastric juice	2.3±0.3
	Intestinal juice	2.3±0.8

* amount of EPS (μg) in the HPLC-injection volume (30 μl)

Table 2. Proliferation index (PI) of peripheral blood mononuclear cells (PBMC), isolated from six healthy donors, co-cultured for 4 days in presence of the ropy *Lactobacillus paraplantarum* BGCG11 and its non-ropy derivatives NB1, NB4 and NB16, as well as in the presence of three concentrations (1, 10 and 100 μg/ml) of the EPS-CG11 or 0.05 μg/ml of LPS from *E. coli*. Independent one-way ANOVA tests were carried out to compare the PI of each stimulus with respect to that of the control (RMPI medium without stimuli, PI =1).

		PI
Stimuli		**Mean ±SD**
LPS *E. coli*	0.05 μg/ml	0.96±0.30
Lb. paraplantarum	BGCG11	1.12±0.16 **
	NB1	1.14±0.28 *
	NB4	1.23±0.17 ***
	NB16	1.23±0.18 ***
EPS-CG11	1 μg/ml	1.04±0.15
	10 μg/ml	1.00±0.15
	100 μg/ml	1.10±0.26

* $p<0.05$, ** $p<0.01$, *** $p<0.001$

672 **Table 3.** Ratios calculated from the cytokines produced by peripheral blood mononuclear
673 cells (PBMC), isolated from six healthy donors, co-cultured for 4 days in presence of the UV-
674 irradiated ropy *Lactobacillus paraplantarum* BGCG11 and its non-ropy derivatives NB1,
675 NB4 and NB16, as well as in the presence of three concentrations (1, 10 and 100 μg/ml) of
676 the EPS-CG11 and 0.05 μg/ml of LPS from *E. coli*. Independent non-parametric Mann-
677 Whitney for 2-independent samples tests were carried out to compare the ratio obtained for
678 each stimulus with respect to that of the control (RPMI medium without stimuli). Differences
679 with respect to the control are marked with asterisks (* $p<0.05$).

680

	Cytokine ratios (mean ±SD)		
Stimuli	**TNF-α/ IL-10**	**IL-10/ IL-12**	**IL-1β/ IL-12**
RPMI (control)	4.28±3.63	0.89±0.21	2.37±1.61
LPS	1.39±1.13	29.13±17.71 *	325.95±258.92 *
Strain BGCG11	133.58±98.33 *	34.88±38.34 *	1074.51±864.45 *
Strain NB1	271.68±244.21 *	14.91±14.02	510.78±435.98 *
Strain NB4	321.01±322.34 *	11.54±11.85	438.27±473.11 *
Strain NB16	274.94±227.43 *	14.69±14.01	515.35±528.76
EPS-CG11 (1 μg/ml)	1.24±0.23	0.93±0.27	0.89±0.10
EPS-CG11 (10 μg/ml)	1.54±0.47	0.87±0.10	1.47±0.23
EPS-CG11 (100 μg/ml)	1.40±0.61	1.51±0.28 *	9.89±4.48 *

681

682 **Figure legends**

683 **Figure 1**. Survival percentage of the bacterial suspensions made in 10% skimmed-milk of the
684 ropy *Lactobacillus paraplantarum* BGCG11 and its non-ropy derivatives NB1, NB4 and
685 NB16 after chemically simulating the gastrointestinal tract (GIT) transit. GJ: gastric juice, DJ:
686 duodenal juice, IJ: intestinal juice. Within each GIT challenge, columns that do not share the
687 same letter are statistically different ($p<0.05$), accordingly to one-way ANOVA and the LSD
688 mean comparison tests.

689

690 **Figure 2**. Adhesion to the human epithelial intestinal cell lines Caco2, HT29 and HT29-MTX
691 of the ropy *Lactobacillus paraplantarum* BGCG11 and its non-ropy derivatives NB1, NB4
692 and NB16, as well as the reference strain *Lactobacillus rhamnosus* GG. Within each cell line,
693 columns with an asterisk are statistically different ($p<0.05$) from the reference strain GG,
694 accordingly to one-way ANOVA tests.

695

696 **Figure 3**. Cytokines (pg/ml) produced by peripheral blood mononuclear cells (PBMC),
697 obtained from six healthy donors, co-cultured for 4 days in the presence of the ropy
698 *Lactobacillus paraplantarum* BGCG11 and its non-ropy derivatives NB1, NB4 and NB16 at
699 ratio 5:1 (bacteria: cell line) **(a)**, as well as in the presence of three concentrations (1, 10 and
700 100 μg/ml) of the purified EPS-CG11 **(b)**. The LPS from *E. coli* (0.05 μg/ml) was used as
701 positive control. The "box and whiskers" figure represents median, inter-quartile range and
702 minimum and maximum values. The statistical differences ($p<0.05$) between each stimuli and
703 the control (RPMI) were assessed by means of the non-parametric Mann-Whitney test for 2-
704 independent samples, and they are indicated with asterisks.

Figure

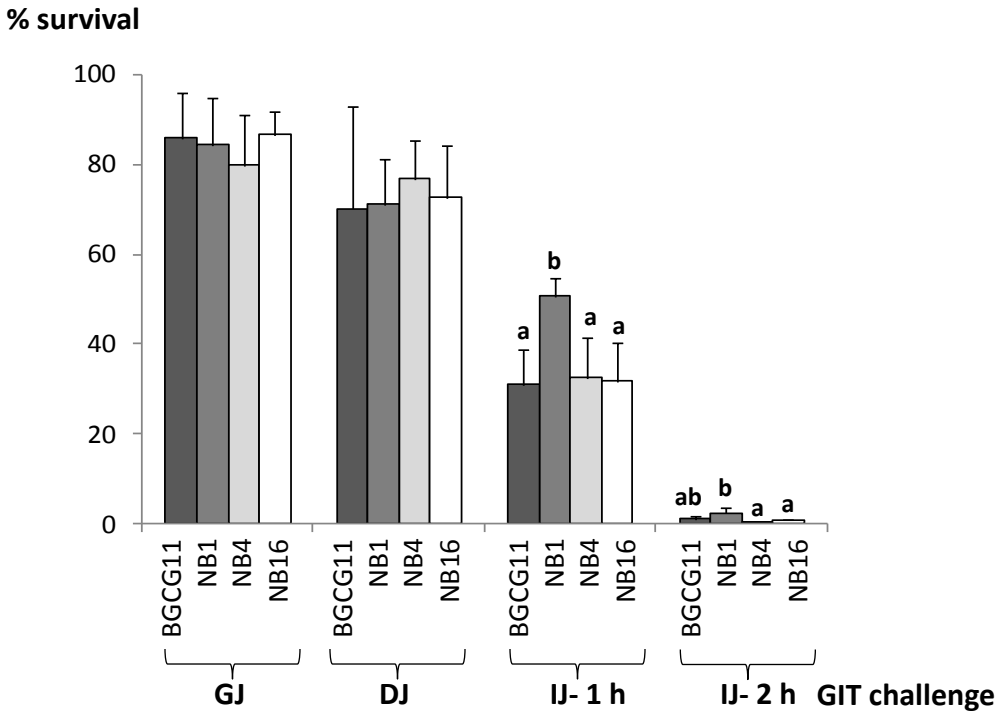


Figure 1

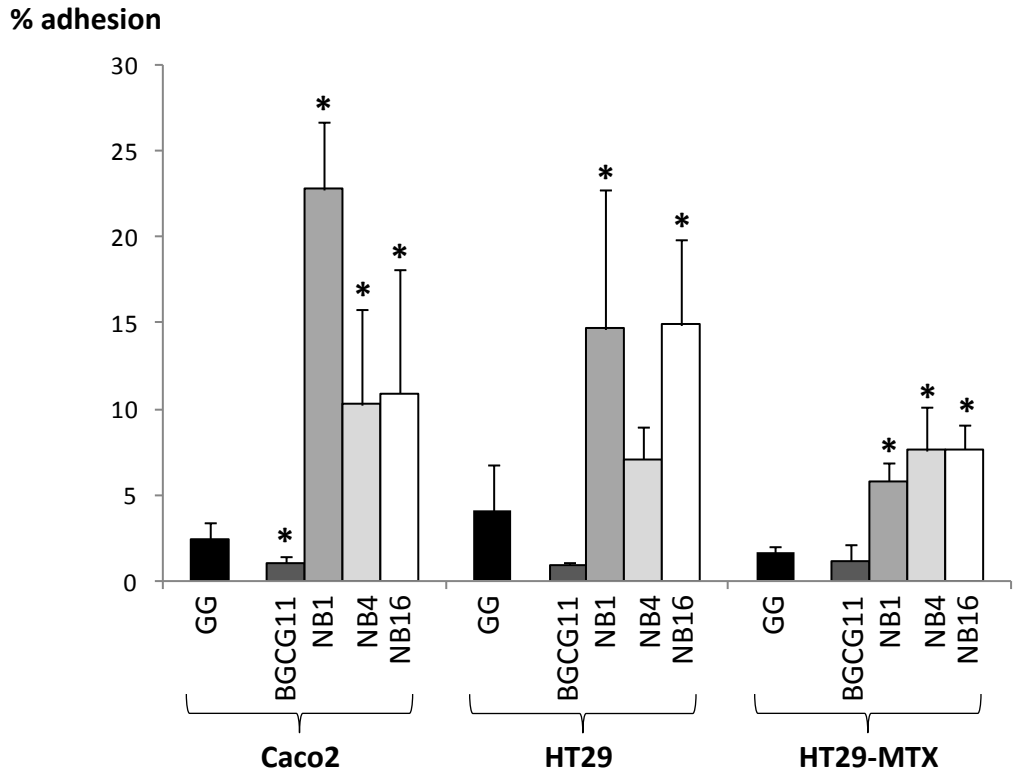

% adhesion
30
25
20
15
10
5
0
GG
BGCG11
NB1
NB4
NB16
Caco2
HT29
HT29-MTX

Figure 2

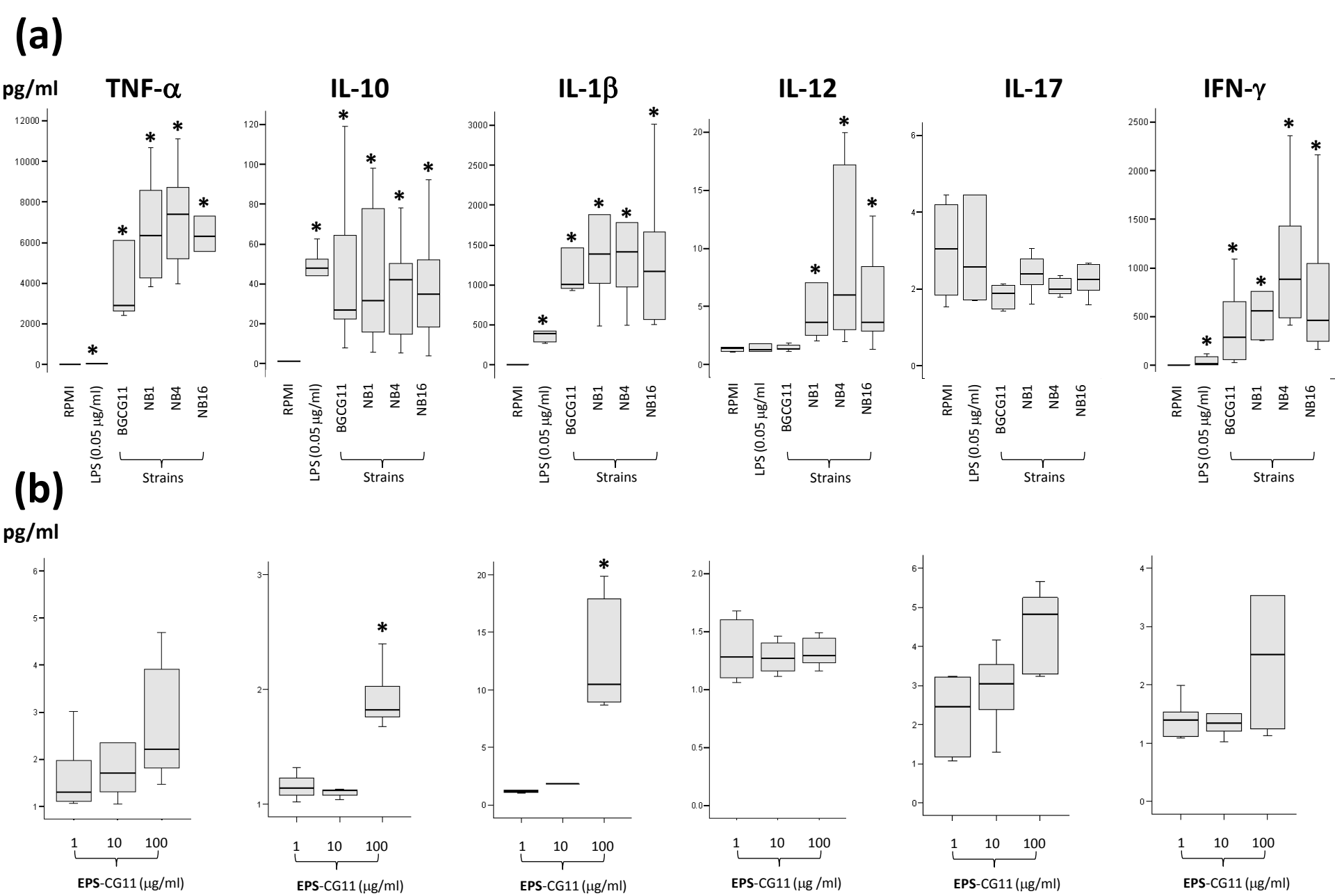

(a)
pg/ml
TNF-α
IL-10
IL-1β
IL-12
IL-17
IFN-γ
RPMI
LPS (0.05 μg/ml)
BGCG11
NB1
NB4
NB16
Strains
(b)
pg/ml
1
10
100
EPS-CG11 (μg/ml)

Fig.3

Supplementary Material

Table S1. Composition of the basal minimum medium (BMM) used to grow the strain *Lactobacillus paraplantarum* BGCG11 for the extraction and purification of its EPS fraction.

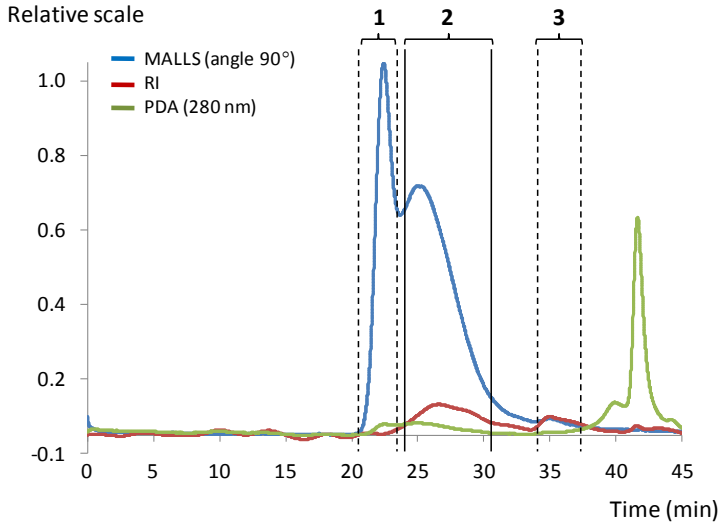
Component	**per L**
Casamino acids*	20 g
Glucose	20 g
Sodium acetate	6 g
Ammonium citrate	1 g
K_2HPO_4	3 g
KH_2PO_4	3 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$MnSO_4 \cdot 1H_2O$	0.032 g
$FeSO_4 \cdot 7H_2O$	0.02 g
Riboflavine	1 mg
Biotine	1 mg
Folic acid	0.1 mg
Nicotinic acid	1 mg
Pantothenic acid	1 mg
Pyridoxine	2 mg
Para Aminobenzoic acid	0.2 mg
Tween 80	1 mg
	pH 6.8

* Casamino acids from Difco, Detroit, MI, USA

Figure S1. Size exclusion chromatography (SEC) analysis of the EPS synthesised by the strain *Lactobacillus paraplantarum* BGCG11, purified according to the procedure described in material and methods section. The lyophilised EPS was dissolved (10 mg/ml) in 0.1 M $NaNO_3$ and the separation (30 µl volume injection) was carried out in the columns and HPLC system described. The figure shows a chromatogram example where the blue line correspond to the multi-angle laser light scattering (MALLS) detector, set in position 11 (angle 90°), the red line correspond to the refraction index (RI) detector and the green line correspond to the photodiode array (PDA) detector set at 280 nm. The results presented in the table are the average of three independent injections. The ASTRA software (Wyatt Europe GmbH, Dembach Germany) was used to analyse results obtained from MALSS detector using the random coil adjust-model.

The peak 1 was not analysed since no signal in the RI was found; probably, the signal detected in the MALLS was due to the aggregation of some polymer particles [peak 1: weight average molar mass (M_w) about $1.7x10^8$ g/mol and weight average radius of gyration (R_w) about 62 nm].

In the peaks 2 and 3, the PDA detector set at 220 nm and 260 nm (detection of peptidic linkage and DNA, respectively) was not giving signal and that at 280 nm (detection of aromatic amino acids) was very weak; thus, we assume that the RI signal of these peaks corresponds to EPS-fractions of polymer. Results of each peak, obtained from data of MALLS and RI, are collected in the table.



	Mean ± SD (n=3)	
	Peak 2	**Peak 3**
Elution time (min)	28.11 ± 0.47	35.83 ± 0.07
Amount (µg)	261.5±2.9	33.3±6.4
Abundance (%)[a]	89.3 ± 22.7	10.7 ± 2.1
Concentration (mg/ml)	8.7±0.1	1.1±0.2
M_w (g/mol)	$2.2x10^6 \pm 0.6x10^6$	$5.6x10^4 \pm 0.8x10^4$
R_w (nm)	42.0 ± 1.7	36.0 ± 3.6
ν (log R_w/log M_w)	0.26 ± 0.02	0.33 ± 0.01

[a] Abundance = amount (µg) of each peak divided by the total amount (peak 2 + peak 3)