Elsevier Editorial System(tm) for LWT - Food Science and Technology

Manuscript Draft

Manuscript Number: LWT-D-18-01461R1

Title: Heteropolysaccharide-producing bifidobacteria for the development of functional dairy products

Article Type: Research paper

Keywords: Bifidobacteria; exopolysaccharide; immunomodulation; zebrafish; technological properties

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Abstract: Bifidobacterium longum INIA P132 and Bifidobacterium infantis INIA P731, isolated from infant-faeces, were investigated in this work. Regarding the probiotic and technological potential of the bifidobacteria, both were resistant to gastrointestinal tract simulated conditions. B. longum showed high survival upon freezing and thawing as well as lyophilisation and was able to grow in milk. B. infantis had higher adhesion capacity to human Caco-2 cells than the commercial probiotic Bifidobacterium animalis BB12 strain. Moreover, both bacteria secrete heteropolysaccharides (HePS) composed of rhamnose, galactose and glucose. In a dextran sodium sulphate-induced enterocolitis model in zebra fish larvae, treatment with each HePS preparation resulted in a decrease of the larval mortality. In addition, the HePS from B. longum immunomodulated in vitro human macrophages treated with the inflammatory Escherichia coli O111:B4 lipopolysaccharide. Thus, both studied bifidobacteria and their HePS have potential beneficial effects on health and thus, to their application in functional foods.

***Cover Letter**

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Universidad del País Vasco
Euskal Herriko Unibertsitatea

KIMIKA APLIKATUAREN SAILA
DEPARTAMENTO DE QUÍMICA APLICADA

San Sebastián, November 17, 2018

Dear Editor:

I am now pleased to send you the new version of our manuscript "Heteropolysaccharide-producing bifidobacteria for development of functional dairy products", which has been revised.

Therefore, we would appreciate if you accept to take in consideration this manuscript to assess its suitability for its publication in LWT-Food Science and Technology.
We look forward for your response.

Kind regards,

Prof. María Teresa Dueñas Chasco

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***Detailed Response to Reviewers**

Comments:
Line 313. Edition mistake at the beginning of the sentence.

The mistake has been corrected.

Section 3.3. The EPS recoveries are lower than the ones obtained by other authors; there is a factor of 10 between this work and the other ones. This difficulties the use of EPS for industrial applications. This fact must be discussed.

The use of EPS for industrial applications has been discussed. However, the isolation of the EPS is a complex procedure, which can lead to the loss of material through the different stages yielding different amounts of total EPS each time. In addition, the different methods for the EPS isolation can also influence the final yield.

Editor's comments

In Tables 2 and 3, please indicate sample size (N=) in the footnote**

The sample size has been added in each experiment of the tables 2 and 3.

Highlights

1. *B. longum* INIA P132 showed good stability as frozen and freeze-dried culture and was able to grow in milk.
2. *B. infantis* INIA P731 adhered highly to human Caco-2 cells and survived GI conditions.
3. EPS of *B. longum* INIA P132 and *B. infantis* INIA P731 were partially characterised.
4. Both HePS reduced larvae mortality in a DSS-induced enterocolitis zebrafish model.
5. *B. longum* INIA P132 EPS immunomodulated *in vitro* human macrophages treated with LPS.

Heteropolysaccharide-producing bifidobacteria for the development of functional dairy products

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Abstract

Bifidobacterium longum INIA P132 and *Bifidobacterium infantis* INIA P731, isolated from infant-faeces, were investigated in this work. Regarding the probiotic and technological potential of the bifidobacteria, both were resistant to gastrointestinal tract simulated conditions. *B. longum* showed high survival upon freezing and thawing as well as lyophilisation and was able to grow in milk. *B. infantis* had higher adhesion capacity to human Caco-2 cells than the commercial probiotic *Bifidobacterium animalis* BB12 strain. Moreover, both bacteria secrete heteropolysaccharides (HePS) composed of rhamnose, galactose and glucose. In a dextran sodium sulphate-induced enterocolitis model in zebra fish larvae, treatment with each HePS preparation resulted in a decrease of the larval mortality. In addition, the HePS from *B. longum* immunomodulated *in vitro* human macrophages treated with the inflammatory *Escherichia coli* O111:B4 lipopolysaccharide. Thus, both studied bifidobacteria and their HePS have potential beneficial effects on health and thus, to their application in functional foods.

Keywords

Bifidobacteria; exopolysaccharide; immunomodulation; zebrafish; technological properties; adhesion.

Abbreviations

DMEM, Dulbecco's Modified Eagle medium; DSS, dextran sodium sulphate; EDTA, ethylenediaminetetraacetic acid; EPS, exopolysaccharides; EW, embryo water; HePS, heteropolysaccharides; HoPS, homopolysaccharides; HPLC-SEC, high-performance size exclusion liquid chromatography; IR, infrared; LAB, lactic acid bacteria; mTSB, modified tryptic soy broth; LPS, lipopolysaccharide; MEM-Alpha, minimum essential

medium-alpha; p-GTF, priming-glycosyltransferase; PMA, phorbol-12-myristate-13-acetate; PMA-THP-1, THP-1 monocytes differentiated to macrophages with PMA; RCM, reinforced clostridial medium; RPMI, Roswell Park memorial institute medium; RT, room temperature; TEM, transmission electron microscopy; TFA, trifluoroacetic acid.

1. INTRODUCTION

Bifidobacteria are a predominant bacterial group present in the human gastrointestinal tract. They have a long history of safe use in food and as probiotics, because they can protect the host by acting as a barrier against exogenous food-borne pathogens, promote nutrient supply and contribute to maintain normal mucosa immunity (Alp & Aslim, 2010; Ruas-Madiedo et al., 2007, 2009). Some of their beneficial effects on the host's health (anti-tumour, cholesterol-lowering, immunomodulating activity, etc) have been attributed to the exopolysaccharides (EPS) that they produce (Hidalgo-Cantabrana et al., 2014a; Inturri et al., 2017). Bifidobacteria synthesise heteropolysaccharides (HePS) and a molecular approach to determine the mechanism of their synthesis is under investigation (Ferrario et al., 2016; Hidalgo-Cantabrana et al., 2014b; Inturri et al., 2015, 2017; Ruas-Madiedo et al., 2007), but it still remains unclear and seems to differ from one strain to another. *Bifidobacterium* genes involved in this synthesis are organised in clusters, called *eps* clusters, but there is not a consensus in their structural organization, their number and the role of their products. The HePS protect bifidobacteria from the acidity and bile salts during their passage through the gastrointestinal tract and can improve their adherence to the intestinal mucosa (Alp & Aslim, 2010; Fanning et al., 2012). Thus, bifidobacteria are currently used to directly produce their EPS in fermented products to exert their probiotic role after ingestion. However, *Bifidobacterium* strains have very stringent growth requirements. Some of them are very sensitive to oxygen, their growth in milk is poor and not all can survive processes used in the food industry (Roy, 2005). Thus, these characteristics of the *Bifidobacterium* strains must be taken into account when searching for a new probiotic. The aims of the present work were to characterise the structure and functionality of the

EPS produced by two bifidobacteria, and to evaluate the bacterial technological and probiotic properties.

2. MATERIALS AND METHODS

2.1. Bacterial strains and culture conditions

B. longum INIA P132 and *B. infantis* INIA P731, isolated from healthy breast-fed infant faeces (Rodríguez et al., 2012), were selected to be studied on the basis of their ropy phenotype. The commercial probiotic strain *B. animalis* BB12 (Chr. Hansen A/S, Hørshom, Denmark) was used for comparison. All bifidobacteria were routinely cultured on Reinforced Clostridial Medium (RCM) broth (Becton, Dickinson and Company), incubated at 37 ºC for 48 h in an anaerobic atmosphere (anaerobiosis generators, BD GasPak™), and conserved at -80 ºC upon addition of 10% glycerol.

2.2. Amplification by PCR of priming-glycosyltransferase genes (p-GTF)

p-GTF enzymes are involved in the synthesis of EPS and can be encoded by different genes in different strains of *Bifidobacterium* (Hidalgo-Cantabrana et al., 2015). To detect the p-GTF coding genes of the two *Bifidobacterium* strains, their genomic DNA was isolated using the 'Wizard® Genomic DNA Purification kit' (Promega) following the manufacturer's instructions. Two sets of degenerated primers previously designed by Hidalgo-Cantabrana et al. (2015) were used: *cpsD*_F4-*cpsD*_R6 and *rfbP*_F5-*rfbP*_R5. PCRs were performed with Taq polymerase (Invitrogen) and conditions were: 3 min at 94 ^{o}C, 30 cycles of 45 s at 94 ^{o}C, 30 s at 60 ºC or 56 ^{o}C for *rfbP*_F5-*rfbP*_R5 or *cpsD*_F4-*cpsD*_R6 and 50 s at 72 ^{o}C, with a 10 min final step at 72 ^{o}C. The DNA sequence of the resulting amplicons was determined at Secugen (Madrid, Spain). Homologies of the DNA sequence of the amplicons and the inferred amino acid

sequences with the genes and proteins deposited in the data banks were searched with BLAST (www.ncbi.nlm.nih.gov/) and Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/).

2.3. EPS isolation and purification

The bifidobacteria were seeded on RCM agar plates, collecting the polymers from the biomass on the plates' surfaces with ultrapure water (1.5 mL/plate) according to López et al. (2012) with modifications. Briefly, cell suspensions were mixed with one volume of 2 M NaOH and incubated overnight at room temperature (RT) with shaking at 180 rpm. Then, trichloroacetic acid was added at a final concentration of 20% (v/v) and kept 1-2 h at 4 ºC under stirring. Supernatants were collected after centrifugation at 18566 × *g* at 4 ºC (20 min) and pH was adjusted to 6.5 by addition of NaOH. Then, the EPS were precipitated with 3 volumes of cold absolute ethanol, incubating overnight at -20 ºC. The precipitates were sedimented by centrifugation at 18566 × *g* at 4 ºC for 10 min and washed 3 times with 80% (v/v) cold ethanol. Finally, EPS-preparations were dialyzed in 12-14 kDa MWCO membranes (Iberlabo) against deionized water, and freeze-dried.

For further reduction of DNA, RNA and proteins, the freeze-dried EPS were dissolved (1 mg/mL) in a solution with 50 mM Tris-HCl, 100 mM $MgSO_4 \cdot 7H_2O$, pH 7.5, and kept at 70 ºC overnight. To eliminate non-dissolved material, the preparations were centrifuged at 8609 × *g* for 15 min at RT. Then, DNase I and RNase A (both from Sigma-Aldrich) were added to the supernatants at a final concentration of 2.5 μg/mL and 10 μg/mL, respectively, and enzymatic digestions were performed at 37 ºC for 6 h with shaking. Afterwards, the EPS-preparations were deproteinized by: (i) treatment with proteinase K (Sigma) at 30 μg/mL for 18 h at 37 ºC with moderate stirring and (ii) two phenolization processes. The latter were performed by addition of 1 mL

phenol:chloroform:isoamyl alcohol (25:24:1) to each EPS solution, vortex for 7 min, centrifugation at 8609 × *g* at RT for 5 min and recovery of the aqueous phases containing the EPS. Afterwards, the EPS preparations were treated with one volume of chloroform:isoamyl alcohol (24:1), vortexed for 7 min and fractionated as indicated above. Finally, samples were dialyzed and freeze-dried. Lyophilized EPS were dissolved in ultrapure water (0.1 mg/mL) and concentration was estimated from the neutral carbohydrate content, determined by the phenol-sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) using glucose as standard. Contaminant DNA, RNA and protein contents were determined in EPS suspensions at 1 mg/mL using specific fluorescent staining kits and the Qubit®2.0 fluorometric detection methods (ThermoFisher Scientific).

2.4. Detection of EPS by electron microscopy

To detect EPS by transmission electron microscopy (TEM), the bifidobacteria were grown in RCM broth. Aliquots (100 µL) of the cultures were centrifuged (5 min, 15700 x *g* at 4 ^{o}C) and the sediments were resuspended in 100 µL of deionized water. For visualisation, bacterial suspensions (50 µL) were processed as previously described (Zarour et al., 2017), with some modifications. Briefly, after the grids were discharged, they were placed facedown over a droplet of each suspension for 15 s and in the negative staining step, the uranyl acetate concentration used was reduced to 1% (w/v). Samples were examined in the Electron Microscopy Facility at the Biological Research Centre (CIB, Madrid, Spain).

2.5. EPS characterization

Neutral sugar composition and linkage types were determined as previously described (Notararigo et al., 2013). The presence of N-acetyl, carboxyl, phosphate or sulphate groups and the α- or β-anomeric configuration of the monosaccharides, as well as the average molecular weight (M_w) of the EPS, were assessed by infrared (IR) spectroscopy and high-performance size exclusion liquid chromatography (HPLC-SEC), respectively, as previously described (Ibarburu et al., 2015).

2.6. *In vivo* protective effect of EPS in a dextran sodium sulphate (DSS)-induced enterocolitis model of zebrafish larvae

Zebrafish embryos were obtained from wild-type adult zebrafish (*Danio rerio*, Hamilton 1822), bred and maintained in the AZTI Zebrafish Facility (REGA number ES489010006105; Derio, Spain) as previously described (Russo et al., 2015) following standard conditions (Sullivan & Kim, 2008). All experimental procedures were approved by the Regional Animal-Welfare Body. Embryos were recovered and cleaned with embryo water (EW; $CaCl_2$ at 294 mg/mL, $MgSO_4 \cdot 7H_2O$ at 123.3 mg/mL, $NaHCO_3$ at 63 mg/mL and KCl at 5.5 mg/mL) and maintained in EW supplemented with methylene blue 0.01% (w/v) at 27 °C. Pools of 20-30 embryos of 1-day post fertilization (dpf) were distributed in Petri dishes containing EW supplemented with the corresponding EPS (150 µg/mL), and incubated at 27 ^{o}C. Co-treatment with the EPS and 0.8% (w/v) of DSS (dextran sodium sulphate, Mw 6,500−10,000 Da, Across Organics) extended from 4 dpf to 7 dpf. Treatments were replaced daily, and the dead larvae were counted at 5, 6 and 7 dpf. Plates in which only DSS was added were used as positive controls of mortality.

To evaluate if the attenuating effect was dose-dependent, the same protocol was performed assaying three different concentrations (50, 100 and 150 µg/mL) of the EPS produced by *B. infantis* INIA P731 strain. Tests were done in triplicate in two independent experiments.

2.7. THP-1 cell line culture and immunomodulation assay

The human monocytic cell line THP-1, obtained from the CIB cell bank, was used for the immunomodulation assay. First, human monocytic THP-1 cells were differentiated to macrophages by treatment with phorbol-12-myristate-13-acetate (PMA), which results in their inability to proliferate (Kohro et al., 2004). Then, PMA-THP-1 cells were treated with the lipopolysaccharide (LPS) of *E. coli* O111:B4 to induce an inflammatory response and the EPS were tested as previously described (Zarour et al., 2017). Each EPS was tested in triplicate in two independent experiments.

2.8. Survival to simulated gastrointestinal conditions

Bifidobacterial survival to gastric and intestinal (GI) conditions was tested based on Haller et al. (2001) and consisted in consecutive exposure of bacterial suspensions to phosphate-buffered saline to pH 3 (adjusted with HCl) and to bile salts (Oxoid) at 1.5 g/L. Each step was performed over 1 h at 37 °C and anaerobic atmosphere. Experiments were performed in duplicate and viable cell population variation was determined by plate counting on RCM agar.

2.9. Biofilm formation

Bifidobacteria grown on RCM agar were resuspended in mTSB (tryptic soy broth, (Biolife), supplemented with 20 g/L of bacto proteose-peptone (Oxoid)). This

suspension was used to inoculate (10%) either mTSB or mTSB supplemented with 0.2% oxgall (Oxoid), and each inoculated broth was loaded into the wells of polystyrene microtiter plates (Nunc 167008) and incubated at 37 ºC for 24 h under anaerobic conditions. Biofilm formation was assessed by the crystal violet method (Lebeer et al., 2007). Control wells with non-inoculated broth were used as blanks and negative controls. Each strain and treatment were tested in at least three independent experiments, each with eight biological replicates.

2.10. Caco-2 cell culture and adhesion assays

The Caco-2 human enterocyte cell line, obtained from the cell bank at CIB, was seeded in 96-well tissue culture plates (Falcon Microtest^{TM}, Becton Dickinson) at a final concentration of 1.25 x 10^5 cells/mL and grown as monolayers of differentiated and polarised cells for 15 days. Cell concentrations were determined as previously described (Garai-Ibabe et al., 2010).

For adhesion experiments, late exponential-phase cultures of the bifidobacteria were diluted in a final volume of 1 mL of DMEM (Invitrogen), to give 1.25 × 10^5 colony forming units (cfu)/mL, and added to Caco-2 cells (ratio 1:1) in a final volume of 0.1 mL per well. After incubation for 1 h at 37 ºC and 5% CO_2, un-adhered bacteria were removed and the cell-associated bacteria quantified after platting onto RCM plates, as previously described (Nácher-Vázquez et al., 2017). All adhesion assays were conducted in triplicate, with two biological replicates in each.

2.11. Technological properties

Survival of frozen bifidobacterial cultures was measured after 21 days of storage at -80 ºC. The strains were grown in RCM for 48 h at 37 ºC in anaerobic conditions and

glycerol was added as cryopreservant to a final concentration of 5% (w/v). Viable cell population was determined by plate counting on RCM agar before and after the process.

For freeze-drying survival assays, bacteria were grown, collected and resuspended in reconstituted skimmed milk (10% w/v; Central Lechera Asturiana) as protective medium, aliquoted into cryotubes and frozen at -80 °C for 24 h. Subsequently, aliquots were lyophilized and stored at 5 °C for 21 days. Freeze-dried bifidobacteria were reconstituted using peptone water and viability was determined by plate counting in RCM agar.

Growth and survival in milk was tested by inoculating the bifidobacteria in reconstituted skimmed milk (10% w/v), incubating in anaerobic conditions at 37 °C for 24 h. Changes in bifidobacteria levels were assessed by plate counting on RCM agar.

Survival in milk under refrigerated conditions was performed by collecting the bacteria grown on RCM agar, resuspending them in skimmed milk and storing the suspensions at 5 °C. Viable cell population was determined by plate counting on RCM agar and checked at 14 and 28 days.

2.12. Statistical analysis

The bacterial adhesion to Caco-2 cells was evaluated by two-way analysis of variance (ANOVA). For tests of EPS immunomodulation of THP-1 cells the SAS 9.4 software (SAS Institute Inc.) applying the T-Student test to assess the significance of the addition of the lipopolysaccharide (LPS) from *E. coli* O111:B4 and then, Dunnett´s test to evaluate the significance of the differences between samples and controls. The SPSS-PC 24.0 Software (SPSS Inc) was used for zebrafish larvae experiments, subjecting data to a one-way ANOVA followed by a *post hoc* Dunnett's T3 test. Results of survival to gastrointestinal conditions and biofilm formation tests were subjected to ANOVA

analysis using a general linear model (GLM) and means' comparison was carried out by Tukey's test, performed using SPSS Statistics 22.0 software (IBM Corp.)

3. Results and Discussion

3.1. Detection of EPS by electron microscopy

B. longum INIA P132 and *B. infantis* INIA P731 were selected for use in a former work because of their mucous and ropy phenotype. This characteristic has been related to the production of EPS (Ruas-Madiedo & de los Reyes-Gavilán, 2005; Torino, Font de Valdez, & Mozzi, 2015). Accordingly, analysis of bifidobacterial cultures by TEM revealed cells with EPS attached to them as well as un-attached EPS (Fig. 1B). Representative examples of *B. infantis* INIA P731 suspensions are depicted in Figs. 1A and 1B. Bifidobacteria are pleomorphic, thus, their shape varies depending on the strain and the growth medium adopting conventional rod or bifurcated 'Y'/'V' morphologies (Biavati et al., 2000; Hidalgo-Cantabrana et al., 2014b). As observed in Figs. 1C and 1D, the two bifidobacteria analysed have the "Y" morphology.

3.2. Detection of genes encoding the priming-glycosyltransferase

It has been reported that a p-GTF enzyme catalyses the first step of the synthesis of the *Bifidobacterium* HePS, by transferring a sugar-1-phosphate to a lipophilic carrier molecule embedded in the bacterial membrane (Ferrario et al., 2016). Moreover, two genes included in *eps* clusters, *cpsD* and *rbfP*, which encode putative p-GTF, have been detected in bifidobacteria and are annotated in the data banks as 'galactosyl-transferase' and 'undecaprenyl-phosphate sugar phosphotransferase', respectively. The two proteins only have homology at their C-terminal region, which includes the catalytic domain, and are highly conserved in bifidobacteria, which harbour one or both coding genes

328 depending on the strain. Thus, the differences between the amino acid sequences of the
329 p-GTF could be due to a domain responsible for the sugar specificity of each enzyme,
330 located at their N-terminal regions (Hidalgo-Cantabrana et al., 2014b).
331 The PCR amplification of the 3´-region of the p-GTF coding genes with two different
332 pairs of oligonucleotides produced only a high yield of one of the expected amplicons
333 for each strain: the 373 bp DNA fragment of *rfbP* for *B. infantis* INIA P731 and the 301
334 bp DNA fragment of *cpsD* for *B. longum* INIA P132 (Fig. 2A). The determination of
335 the DNA sequence of the specific amplicons and the homology search with the BLAST
336 program confirmed that *B. infantis* INIA P731 harbours a gene with 99%-90% identity
337 to the *rfbP* of several *Bifidobacterium breve* and *B. longum* strains as well as that *B.*
338 *longum* INIA P132 carries a gene with 100%-96% identity to the *cpsD* of other *B.*
339 *longum* strains. Also, the sequenced region of the genes from *B. infantis* INIA P731 and
340 *B. longum* INIA P132 showed between them an identity of 70.1% indicating that the
341 DNA region encoding the catalytic domain of the p-GTF protein has evolved from a
342 common ancestral gene. Finally, the alignment of the predicted amino acid sequence of
343 the two gene products (Fig. 2B) revealed a fragment of the C-terminal region of the
344 proteins highly-conserved in p-GTF (73.9% identity) (Hidalgo-Cantabrana et al., 2015;
345 Ruas-Madiedo et al., 2007), with a glutamate (E) described as a probable catalytic
346 residue in the p-GTF of *Lactococcus lactis*, and a tyrosine (Y) specific for
347 galactosyltransferases (Ruas-Madiedo et al., 2007; Van Kranenburg et al., 1999). These
348 data, together with the observation of mucoid colonies with ropy phenotype on RCM-
349 agar plates and the visualization of EPS by TEM, encouraged us to isolate the EPS
350 produced by the two strains.
351
352

3.3. Isolation and partial characterization of the EPS

The EPS were separated from biomass harvested from RCM-agar plates, recovering 0.5-0.8 mg and 0.2-0.3 mg of the polymers per plate for *B. longum* INIA P132 and *B. infantis* INIA P731, respectively. These recoveries are much lower than ~~within the~~ those ranges obtained previously for other bifidobacteria: ~~ranges~~ 0.78-4.34 mg EPS/plate for different *Bifidobacterium* species (Salazar et al., 2008) and 3.6-3.8 mg/plate reported for *Bifidobacterium longum* NB667 ~~previously described but lower than the~~ (Salazar et al., 2012). The isolation of the EPS from cultures of bifidobacteria is complex and can lead to a different recovery, and then to different yields, depending on the method used. Therefore, it would be very difficult for these two EPS to be produced as prebiotics by the food industry, due to the low amount recovered. However, their synthesis *in situ* by the producing bifidobacteria in different fermented food would be more suitable to exert their beneficial effects.

The crude EPS precipitates contained residual amounts of DNA (0.01-0.03%), RNA (0.026-0.04%) and protein (1.5-2.5%) and, after enzymatic elimination of nucleic acids and deproteinization these values were further reduced to <0.01%, <0.02% and <1%. Higher protein levels (1.9-8.9%) were reported for EPS preparations from other intestinal *Bifidobacterium* strains (Ruas-Madiedo et al., 2010; Salazar et al., 2008).

Both EPS preparations were partially characterised to analyse their similarities and/or differences. IR spectra (Fig. 3A) showed the typical profile of polysaccharides. Absorption bands in the region of 3400, 1400 and 1060 cm^{-1}, correspond to the hydroxyl stretching vibration (Salazar et al., 2012). Those around 2925-2930 cm^{-1} are due to C-H stretching (Ahmad et al., 2010; Han et al., 2014; Xu et al., 2011), and signals in the 1860-1660 cm^{-1} region result from carbonyl stretching (Salazar et al., 2012; Xu et al., 2011). Absorption between 1200-1000 cm^{-1} is due to the stretching

vibration of glycosidic linkage (C-O-C) and C-O or C-O-H groups (Ahmad et al., 2010; Han et al., 2014). The fingerprint region (<1500 cm^{-1}), characteristic of each molecule (Xu et al., 2011), presented a band around 1020 cm^{-1}. In the anomeric region, 950-700 cm^{-1}, (Xu et al., 2011) a band at 895 cm^{-1} was observed in the spectrum of the crude *B. infantis* INIA P731 EPS, indicating its predominance of β anomers (Ahmad et al., 2010), while no bands were observed in this region for the EPS produced by the other strain studied, highlighting a first difference between them.

Regarding monosaccharide composition, both strains contained glucose, galactose and rhamnose, although in different proportions: 16:10:1 for *B. longum* INIA P132 and 28:10:8 for *B. infantis* INIA P731. These results correlate with the fact that bifidobacteria synthesise various HePS composed of these three monosaccharides (Hidalgo-Cantabrana et al., 2015; Kohno et al., 2009; Mozzi et al., 2006; Salazar et al., 2009). As several repeating units have been reported for bifidobacterial HePS, the types of *O*-glycosidic linkages in the two HePS preparations were investigated by methylation analysis. The results, depicted in Table 1, revealed the same seven predominant types of residues in both of them. The majority of glucose molecules were present as linear-chain units 1,4-linked, and at terminal positions of the side-chains, and also low percentages of 1,6 and (1,4,6)-linked glucopyranose were also detected. The relative proportion of the glucopyranose residues in both samples was similar. On the contrary, as already expected from monosaccharide analysis, the amount of rhamnose and galactose units differed considerably in the two HePS analysed. Rhamnose was exclusively attached to its adjacent residue in the polysaccharide chain by 1,3 linkages, representing almost 20% in the HePS from *B. infantis* INIA P731. Galactose was found in branching points, as a pyranose substituted at *O*-3 and *O*-6, and as linear-chain units 1,4-linked or 1,5-linked, representing about 30% of the HePS from *B. longum* INIA

403 P132. Unfortunately, this uncertainty could not be resolved with this methodology since
404 both residues are transformed into the same partially methylated alditol acetate: 1,4,5-
405 tri-*O*-acetyl-2,3,6-tri-*O*-methyl galactose. The presence of either 1,4-Gal*p* and 1,5-Gal*f*
406 in HePS from bifidobacteria has been reported (Hidalgo-Cantabrana et al., 2014b). In
407 addition, the backbone of the extracellular HePS from *B. longum* JLB05 contains α-
408 (1,4)-Gal*p*, and no galactofuranose (Kohno et al., 2009). Moreover, pyruvic acid was
409 reported in these HePS, and this or another organic acid could be present in the
410 polymers analysed in the current work, since a slight carbonyl band (1736 cm^{-1}), more
411 evident in the sample from *B. longum* INIA P132, was observed in the IR spectra (Fig.
412 3A). Analysis of the HePS preparations from the two bifidobacteria by analytical
413 HPLC-SEC (Fig. 3B) indicated that they contained two fractions of different M_w, as
414 reported before by other authors (Ruas-Madiedo et al., 2010; Salazar et al., 2008; Xu et
415 al., 2011). *B. infantis* INIA P731 contained a major peak of M_w about 1.9×10^5 Da and a
416 smaller one of 1.2×10^4 Da. In *B. longum* INIA P132 the two polysaccharides were in
417 similar amounts, with the peak of high M_w being *ca.* 1.0×10^6 Da and the other of
418 1.3×10^5 Da. These M_w are in the range of those of other HePS produced by
419 bifidobacteria (Kohno et al., 2009; Xu et al., 2011).
420
421 **3.4. Immunomodulatory activity of HePS from bifidobacteria**
422 The EPS seem to play a role in counteracting the inflammatory effect produced by
423 probiotic strains, thus, preventing them from being attacked by the immune system
424 (Schiavi et al., 2016; Yasuda, Serata, & Sako, 2008). Moreover, the EPS' physicochemical differences (negative charges, high or low molecular weight, etc) seem
426 to affect their immunostimulation capacity.
427 Thus, we evaluated the immunomodulatory activity of the HePS of the two
428 bifidobacteria *in vitro*. To this end, human PMA-THP-1 macrophages were treated with

429 the HePS preparations and with *E. coli* LPS to induce an inflammatory response. The
430 levels of the TNF-α inflammatory and IL-10 anti-inflammatory cytokines secreted by
431 treated PMA-THP1 were compared to those secreted by macrophages either untreated
432 or exposed only to LPS, which were used as controls (Fig. 4). All treatments induced
433 the production of both cytokines (Fig. 4A and 4B). The levels of the individual
434 cytokines were similar for treatments with LPS alone and in co-treatment with HePS
435 from *B. infantis* INIA P731, but in the presence of the HePS from *B. longum* INIA P132
436 a significantly higher production of TNF-α and IL-10 ($p<0.05$) was observed. The TNF-
437 α/IL-10 ratios calculated for the challenged macrophages were consistent with an
438 inflammatory response (Fig. 4C) but, even though the differences among the ratios in
439 the three treatments were not statistically significant, an anti-inflammatory trend of the
440 HePS tested can be observed in this model (Fig. 4C). According to Hidalgo-Cantabrana
441 et al. (2014b), high molecular mass EPS show a lower capacity to influence the release
442 of anti- and pro-inflammatory cytokines by macrophages. However, these results could
443 also be explained by an over-stimulation of the eukaryotic cells provoked by the LPS,
444 which can mask the effect of the EPS.
445
446 **3.5. Protective effect of bifidobacterial HePS in an *in vivo* zebrafish model of**
447 **enterocolitis**
448 To assess the anti-inflammatory effect of the HePS preparations isolated from the two
449 bifidobacteria, we used an *in vivo* enterocolitis model of zebrafish, induced by the
450 chemical agent DSS. The data in Fig. 5A show that both HePS reduce the negative
451 effects caused by the DSS. Around 80% of the larvae died upon 7 days of exposure to
452 0.8% DSS, while a reduced mortality was observed in a co-treatment with the *B.*
453 *longum* INIA P132 HePS preparation ($51.7 \pm 3.6\%$, $p<0.05$) and, more notably, with

454 that of *B. infantis* INIA P731 (26.7 ± 2.2%, $p<0.05$). Considering that polymers of *B.*
455 *infantis* strain exerted the highest protection against DSS damage, we used this mixture
456 to test if the effect was dose-dependent. Three different HePS concentrations (50, 100
457 and 150 µg/mL) were evaluated and, as expected, the highest reduction in larvae
458 mortality (31.68 ± 1.32%, $p<0.01$) was achieved when 150 µg/mL of the EPS were
459 administered, confirming a dose-dependent effect (Fig. 5B). The mechanism by which
460 these polymers reduce mortality is still unknown. However, according to the
461 experiments performed in a DSS-induced colitis mouse model with the strain *B.*
462 *animalis* subsp. *lactis* Balat_1410^{S89L} (Hidalgo-Cantabrana et al., 2016), they could
463 present an anti-inflammatory activity mediated by the induction of Treg cells in
464 mesenteric lymphoid nodes, rather than acting as a physical barrier avoiding DSS
465 action. However, further immunological assays should be performed to confirm this
466 hypothesis.
467
468 **3.6. Survival of bifidobacteria to simulated gastrointestinal conditions**
469 The probiotic potential of the two EPS-producing bifidobacteria was first assessed by
470 measuring their survival under *in vitro* GI conditions (Table 2A). The two
471 *Bifidobacterium* strains showed good stability to the GI conditions, with reductions
472 around 0.3 log cfu/mL, although their survival was lower than that of *B. animalis* BB12.
473 This tolerance to acidic pH and bile salts exhibited by the two strains suggests their
474 potential to survive passage through the GI tract and to reach the intestine at sufficient
475 levels to exert their effects.
476 **3.7. Biofilm formation by bifidobacteria**
477 The HePS involved in biofilm formation can affect colonisation and survival of
478 bifidobacteria in the gut (Hidalgo-Cantabrana et al., 2014b). In the present work, *B.*

infantis INIA P731, in the absence of oxgall, showed the better result for biofilm formation of the two EPS-producing bifidobacteria (Table 2B). Neither of the two strains increased the biofilm formation in the presence of oxgall, as has been described before for certain strains of bifidobacteria and lactobacilli (Ambalam et al., 2014; Lebeer et al., 2007) and both new bifidobacterial strains showed lower biofilm formation than *B. animalis* BB12 (Table 2B).

3.8. Analysis of the adhesion ability of the bifidobacteria

Adherence to human epithelial cells is one of the *in vitro* tests listed in the guideline for the evaluation of probiotics in food (FAO/WHO, 2002). Thus, the adhesion capacity of *B. longum* INIA P132 and *B. infantis* INIA P731 was assessed using the enterocyte-like Caco-2 cell line. Some authors have reported that the presence of EPS in the surface of bacteria has a negative effect on their adhesive properties (Castro-Bravo et al., 2017; López et al., 2012; Nácher-Vázquez et al., 2017), while others described the production of these polymers as useful for probiotics to interact with eukaryotic cells (Fernández de Palencia et al., 2009; Garai-Ibabe et al., 2010; Živković et al., 2016). In this work, in accordance with the results obtained for the biofilm formation, *B. longum* INIA P132 showed low binding capacity to the enterocytes, whereas adhesion of *B. infantis* INIA P731 to the epithelial intestinal cells was significantly high ($p<0.05$), exceeding *B. animalis* BB12 levels (Table 2C).

3.9. Technological properties of *Bifidobacterium* strains

The development of a functional food containing live probiotic cultures requires that the probiotic strain is able to survive both the manufacturing process and during the shelf life of the product. A preliminary technological characterization of the two bifidobacterial strains was performed by assessing their survival as frozen or freeze-

505 dried cultures, and their growth and survival in milk under refrigeration conditions. *B.*
506 *longum* INIA P132 showed better stability as frozen and freeze-dried culture than *B.*
507 *infantis* INIA P731 (Table 3). Moreover, *B. longum* was able to grow in milk, while the
508 levels of *B. infantis* INIA P731 decreased in these conditions. This strain showed better
509 stability in milk under refrigerated conditions. According to these results, more research
510 is needed to improve the yield and survival of the strains under the manufacturing
511 conditions and to test their behaviour in different food matrices.
512
513 **4. Conclusions**
514 The mucoid aspect and the ropiness of *B. longum* INIA P132 and *B. infantis* INIA P731
515 colonies suggested them as EPS producers, which was confirmed after obtaining EPS
516 preparations from solid cultures. The polymers were partially characterised as mixtures
517 of HePS with different ratios of rhamnose, galactose and glucose. The same linkage
518 types were detected in both EPS preparations, although their proportions varied.
519 Additionally, the biological activity of these HePS mixtures was studied. Both EPS
520 showed a protective effect to DSS-treated zebrafish larvae. Although not conclusive, an
521 anti-inflammatory tendency was also observed in experiments performed with PMA-
522 THP-1 macrophages. Along with the EPS activities, both strains showed good survival
523 under gastrointestinal conditions, and *B. infantis* INIA P731 displayed higher biofilm
524 formation and adhesion capacity to enterocytes, which is a very remarkable property of
525 probiotic strains. On the other hand, *B. longum* INIA P132 showed better technological
526 suitability. Taken together, these findings suggest that the two strains evaluated, ~~as well~~
527 ~~as their HePS~~, might be used as adjuncts in the food industry and give an added value to
528 fermented products when synthesizing their HePS *in situ*.
529

Declaration of interest

Conflict of interest: none.

Funding

This work was supported by a grant from de Department of Environment, Territorial Planification, Agriculture and Fishing and the project no. IT 866-13 from the Basque Government, by the Spanish Ministry of Science and Innovation (AGL 2015-65010-C3-1-R) and by the Spanish Ministry of Economy and Competitiveness (RM12-00004-00-00 and RTA2013-00029-00-00).

Acknowledgments

We thank Dr. Guillermo Padilla Alonso for his valuable assistance in the bio-statistical analysis. We thank Dr. Stephen Elson for the critical reading of the manuscript.

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Table 1. Linkage types and their proportions (%) in the crude EPS of each strain, deduced from a methylation analysis.

Linkage types	**Proportion (%)**	
	B. infantis INIA P731	*B. longum* INIA P132
Rha*p*-(1-3)	10.3	1.6
Glc*p*-(1-	14.9	22.1
Gal*p*-(1-4)	0.8	15.2
Glc*p*-(1-4)	59.3	39.9
Gal*p*-(1-3)	1.6	0.0
Glc*p*-(1-6)	1.1	2.3
Glc*p*-(1-4,6)	1.2	3.4
Gal*p*-(1-3,6)	10.0	15.7

Table 2. Probiotic characterization of bifidobacterial strains.

Strain	[1]Survival to gastrointestinal conditions	[2]Biofilm formation		[3]Adherence to Caco2 cells
		mTSB	mTSB + 0.2% oxgall	
***B. longum* INIA P132**	-0.32 ± 0.27^{a}	0.02 ± 0.01^{ab}	0.01 ± 0.01^{a}	1.45 ± 0.29^{a}
***B. infantis* INIA P731**	-0.31 ± 0.05^{a}	0.21 ± 0.13^{c}	0.08 ± 0.06^{b}	35.91 ± 5.24^{b}
***B. animalis* BB12**	0.18 ± 0.05^{b}	0.62 ± 0.14^{d}	0.71 ± 0.20^{e}	20.34 ± 5.48^{c}

[1]Survival to gastrointestinal conditions expressed as change in log cfu/mL (mean ± SD). Values with different superscript differ significantly (Tukey test, $p<0.05$). Two independent experiments were carried out with two replications in each (N=4).

[2]Biofilm formation expressed as increment of OD_{570nm} (mean ± SD). Values with different superscript differ significantly (Tukey test, $p<0.05$). Three independent experiments were carried out with 8 replications in each (N=24).

[3]Adhesion to Caco-2 cells, expressed as the percentage of cfu (mean ± standard error (SE)). Values in each column with different superscript differ significantly (Two-way ANOVA, $p\leq0.05$). Three independent experiments were carried out with two replicates in each (N=6).

Table 3. Technological characteristics of the two *Bifidobacterium* strains.

Strain	-80 ºC survival (21 d)	Freeze-drying survival (21 d)	Growth in milk (24 h)	4 ºC storage (14 d)	4 ºC storage (28 d)
***B. longum* INIA P132**	-0.53 ± 0.15	-0.65 ± 0.03	1.52 ± 0.54	-0.92 ± 0.14	-1.70 ± 0.11
***B. infantis* INIA P731**	-1.06 ± 0.15	-1.81 ± 0.26	-0.99 ± 0.21	-0.61 ± 0.10	-1.21 ± 0.05

Data are expressed as change in log cfu/mL^{-1} (mean ± SD) after each procedure. Two independent experiments were carried out with two replicates in each (N=4).

Figure

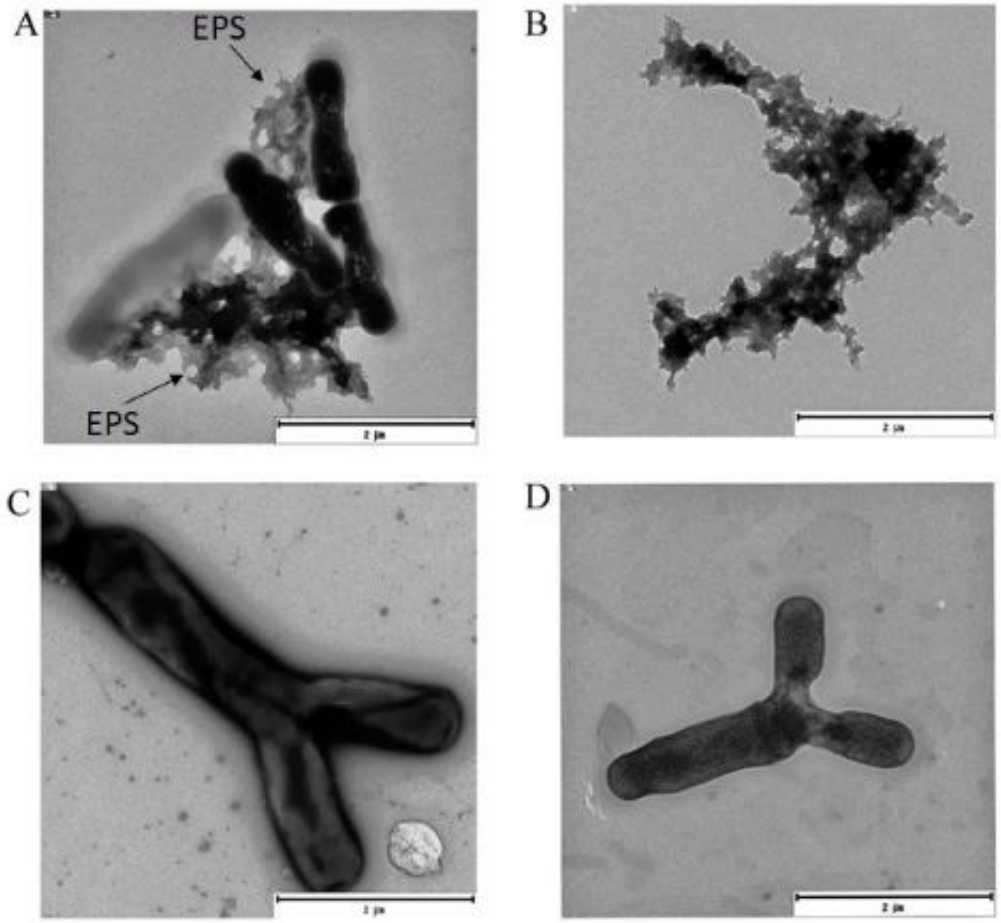

Fig. 1. Visualization of bifidobacteria and their EPS by TEM. B. infantis INIA P731 (A) and its EPS (B). Detection of 'Y' morphology of B. infantis INIA P731 (C) and B. longum INIA P132 (D).

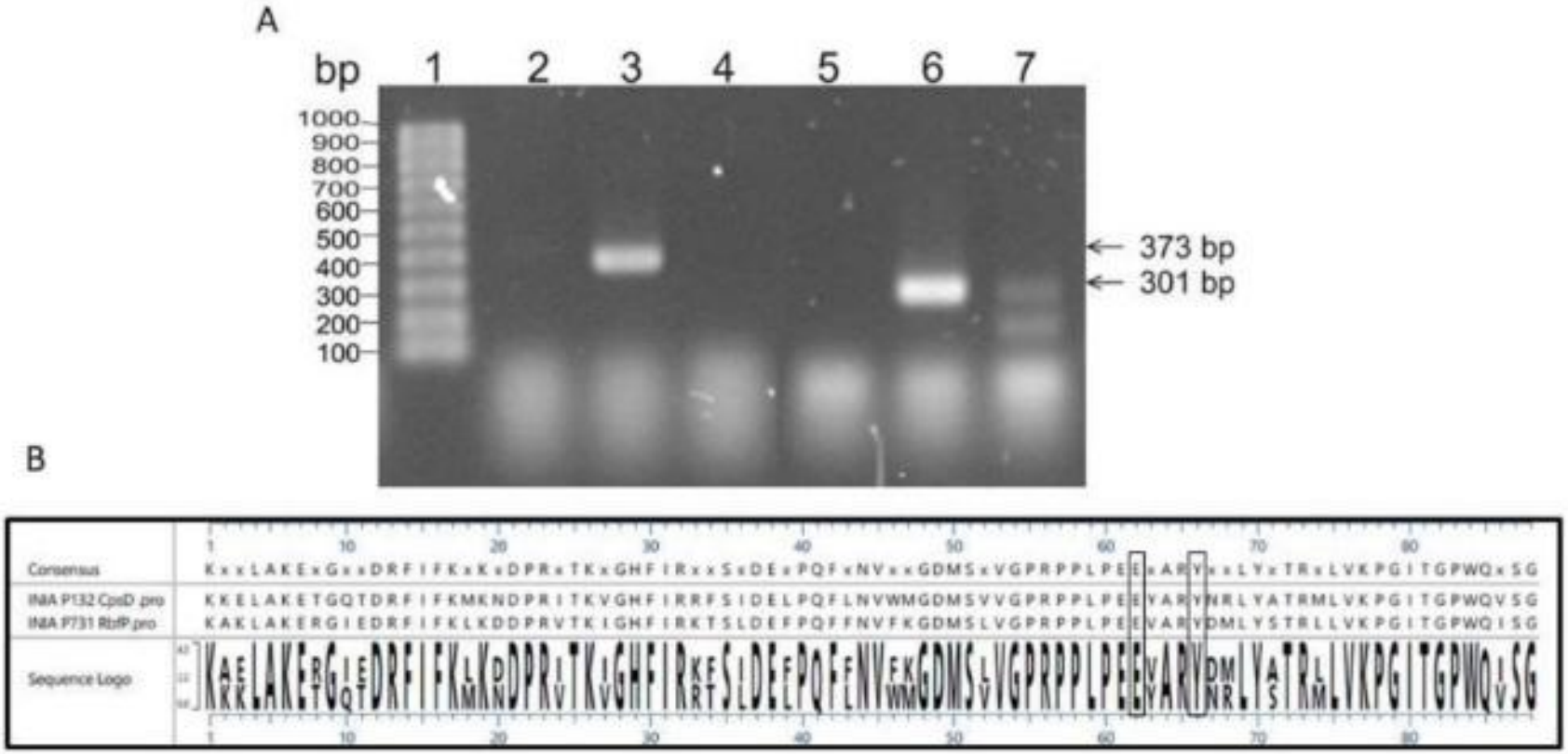

Fig. 2. Genetic analysis of EPS production by *Bifidobacterium* strains. (A) Detection by PCR of *cpsD* and *rbfP* in 1% agarose gel. Lanes: 1, BIORAD Ez Load 100 bp Molecular Ruler; 2, PCR with only *rbfP* F5-R5 primers (negative control); 3, PCR of INIA P731 DNA with *rbfP* F5-R5 primers; 4, PCR of INIA P132 DNA with *rbfP* F5-R5 primers; 5, PCR with only *cpsD* F4-R6 primers (negative control); 6, PCR of INIA P132 DNA with *cpsD* F4-R6 primers; 7, PCR of INIA P731 with *cpsD* F4-R6 primers. (B) Clustal omega alignment and consensus sequences of the inferred amino acid sequences of CpsD from INIA P132 and RbfP from INIA P731 are depicted. Conserved glutamate (E) and tyrosine (Y) residues among p-GTF at the putative catalytic centre are boxed.

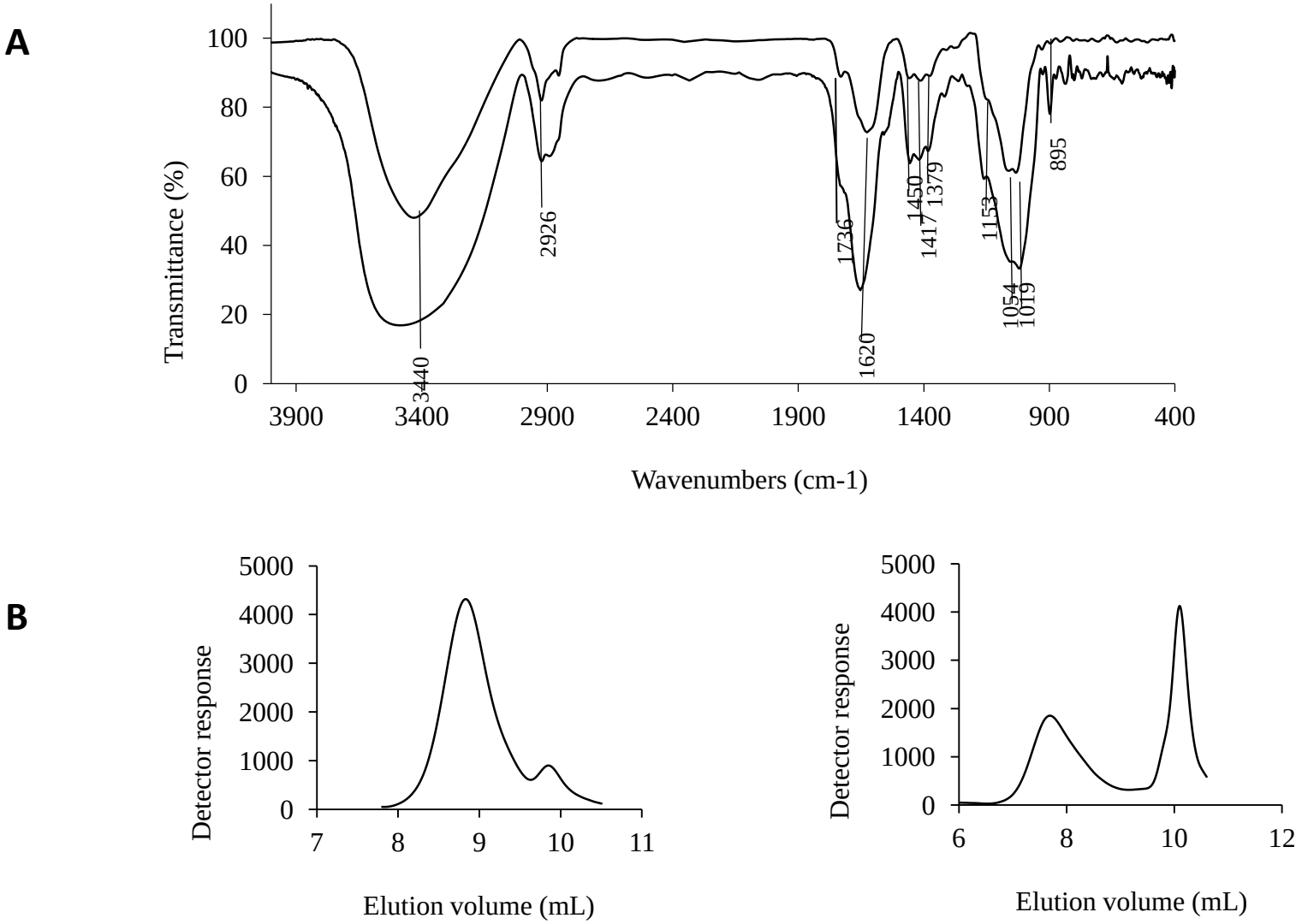

Fig. 3. Physicochemical analysis of bifidobacterial EPS preparations. (A) IR spectra. Up, EPS from *B. longum* INIA P132. Down, EPS from *B. infantis* INIA P731. (B) HPLC-SEC elugrams. Left, EPS fractions from *B. infantis* INIA P731. Right, EPS fractions from *B. longum* INIA P132.

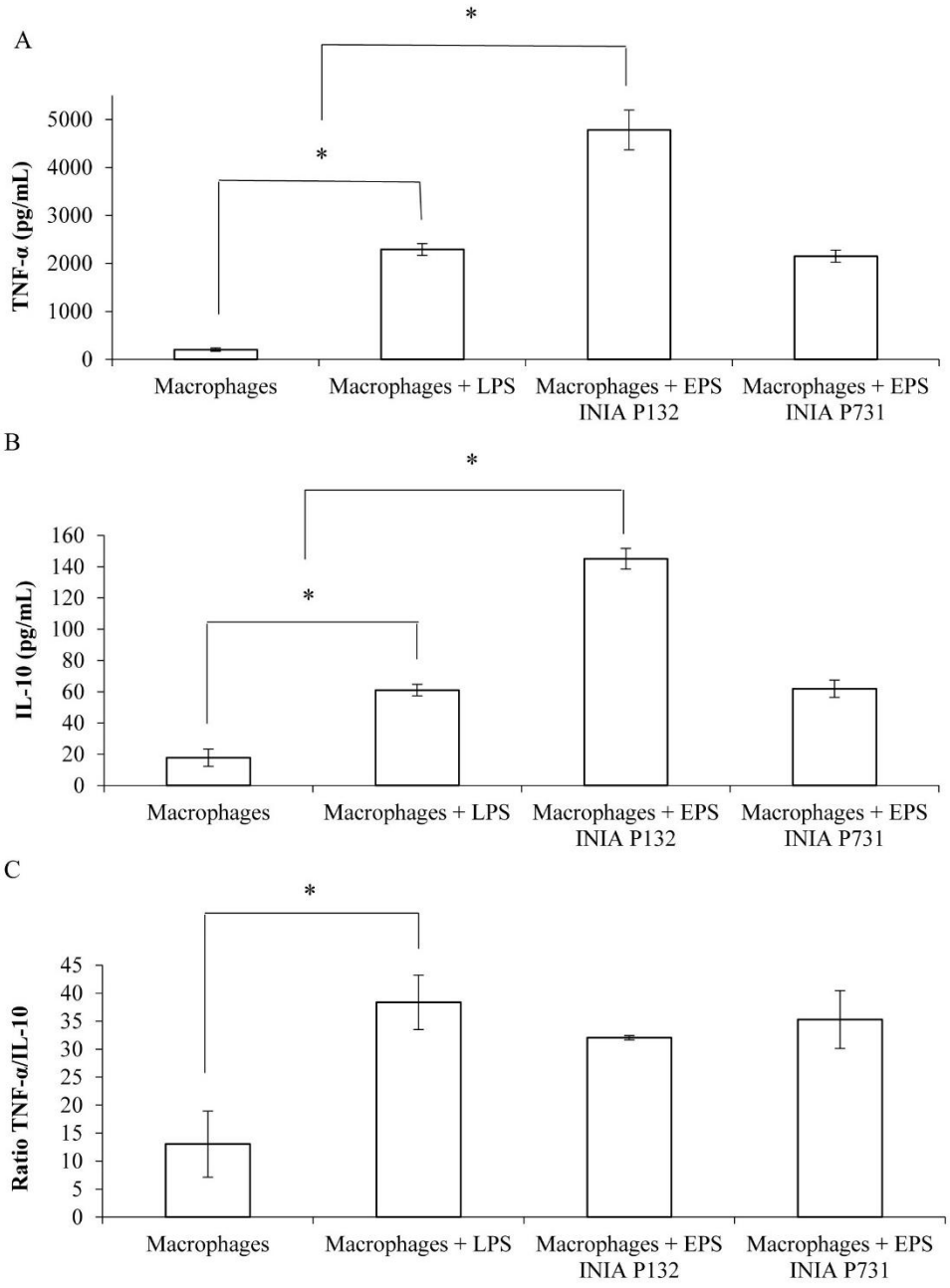

Fig. 4. Evaluation of the cytokines production in the supernatants of THP-1-PMA macrophages after the co-treatment with LPS and the HePS produced by bifidobacteria. Levels of TNF-α (A), IL-10 (B) and ratio TNF-α/IL-10 (C) are depicted. Data were analysed by the T-student's t-test. Then, Dunnett's test was employed to assess the significant differences between the samples and the controls. When significance was $p \leq 0.05$, it was indicated with *.

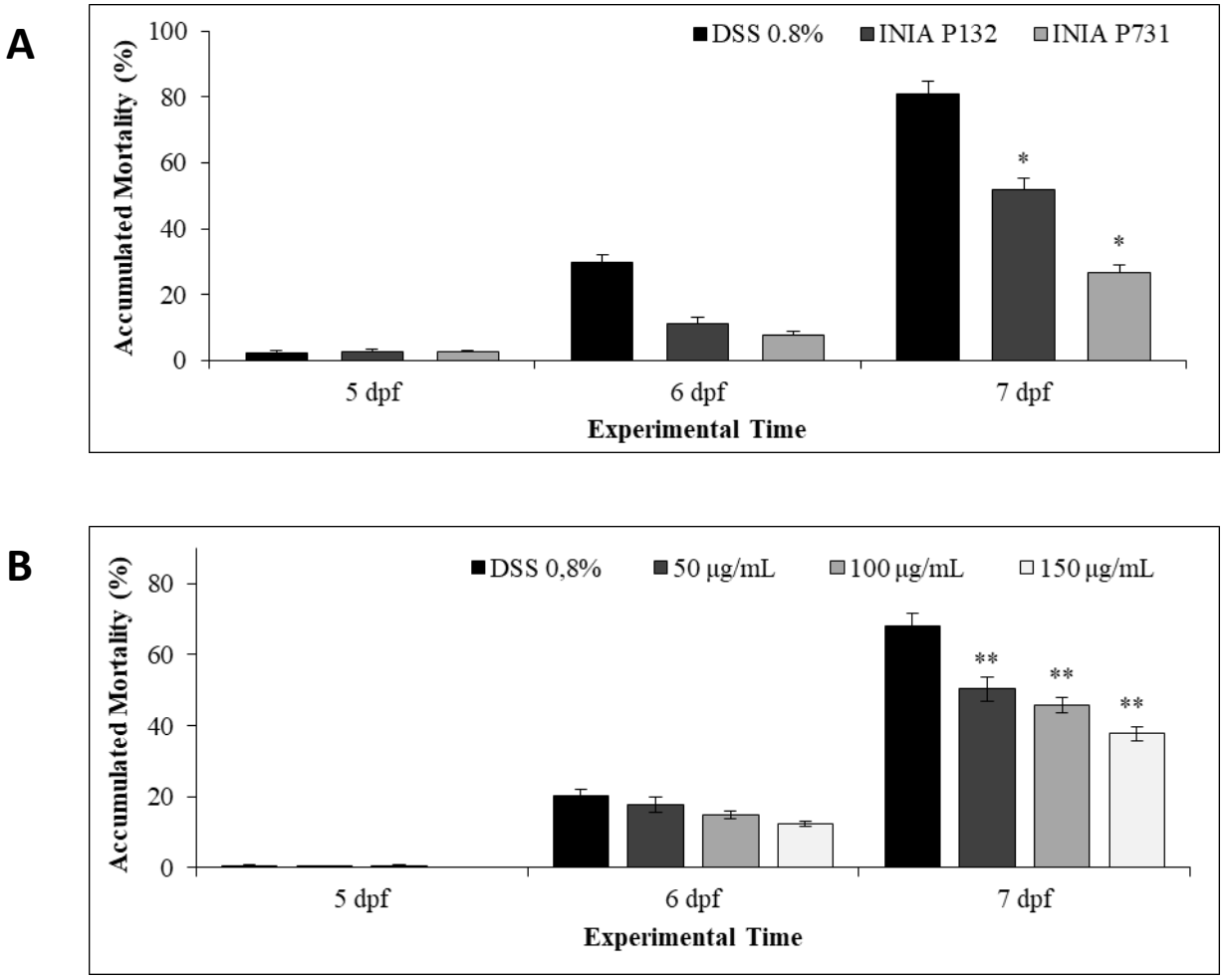

Fig. 5. Protective effect *in vivo* of bifidobacterial HePS in a DSS-induced enterocolitis zebrafish model. (A) Analysis of the mortality of zebrafish larvae treated with DSS 0.8% and HePS of *B. longum* and *B. infantis* strains. (B) Evaluation of dose-dependent effect of HePS produced by *B. infantis* INIA P731 on survival of zebrafish larvae treated with DSS 0.8%. The results are expressed as mean ± standard deviation (SD) and data were subjected to a one-way ANOVA followed by a post hoc Dunnett's T3 test with significances of $p<0.05$ (*) and $p<0.01$ (**).