Characterization of exopolysaccharides produced by Bifidobacterium longum NB667 and its cholate-resistant derivative strain IPLA B667dCo

Journal:	*Journal of Agricultural and Food Chemistry*
Manuscript ID:	jf-2011-04034n.R2
Manuscript Type:	Article
Date Submitted by the Author:	27-Dec-2011

Complete List of Authors:	Salazar, Nuria; Instituto de Productos Lacteos de Asturias, Consejo Superior de Investigaciones Científicas, Microbiology and Biochemistry of Dairy Products Ruas-Madiedo, Patricia; Instituto de Productos Lacteos de Asturias, Consejo Superior de Investigaciones Científicas, Microbiology and Biochemistry of Dairy Products Prieto, Alicia; Centro de Investigaciones Biologicas, Consejo Superior de Investigaciones Científicas (CIB-CSIC), Calle, Luis; Centro de Investigaciones Biológicas, CSIC, Protein Science de los Reyes-Gavilan, Clara; Instituto de Productos Lacteos de Asturias, Consejo Superior de Investigaciones Científicas, Microbiology and Biochemistry of Dairy Products

Title: **Characterization of exopolysaccharides produced by *Bifidobacterium longum* NB667 and its cholate-resistant derivative strain IPLA B667dCo**

Authors: **Nuria Salazar[1], Patricia Ruas-Madiedo[1], Alicia Prieto[2], Luis P. Calle[2], and Clara G. de los Reyes-Gavilán[1*]**

Addresses:

1. Instituto de Productos Lácteos de Asturias-Consejo Superior de Investigaciones Científicas (IPLA-CSIC), Departamento de Microbiología y Bioquímica de Productos Lácteos. Carretera de Infiesto s/n, 33300 Villaviciosa, Asturias, Spain.

2. Centro de Investigaciones Biológicas (CIB-CSIC), Ramiro de Maeztu 9, 28040 Madrid, Spain.

*Clara G. de los Reyes-Gavilán (Tel: 34 985893335; Fax: 34 985892233; E-mail: greyes_gavilan@ipla.csic.es)

ABSTRACT

Bifidobacteria are natural members of the human intestinal microbiota and some strains are being used as probiotics. Adaptation to bile can allow them to increase survival in gastrointestinal conditions, thus improving their viability. *Bifidobacterium longum* NB667 and the cholate-resistant strain *B. longum* IPLA B667dCo produced exopolysaccharides (EPS) that were partially characterized. Analysis by size exclusion chromatography-multiangle laser light scattering indicated that the EPS crude fractions of both strains contained two polymer peaks of different molar mass. Based on chromatographic techniques both peaks appeared to be heteropolysaccharides. The smaller peak was mainly composed of glucose, galactose and rhamnose whose molar ratios and linkage types showed slight variations between the EPS fractions of both strains. The bigger peak consisted of glucose and galactose; the monosaccharide composition was identical in the EPS fractions of the two microorganisms, but their infrared spectra presented some differences regarding compounds other than carbohydrates that seem to be associated to the polymer. Differences in the composition of EPS fractions did not affect the capability of crude EPS from *B. longum* to be fermented by the human intestinal microbiota in fecal batch cultures.

Keywords: *Bifidobacterium,* exopolysaccharide, cholate, SEC-MALLS

INTRODUCTION

Bifidobacteria are natural members of the human intestinal microbiota, in which they occur at concentrations of 10^9 to 10^{11} cells per gram of feces, representing up to 91% of the total gut population in the early stages of life.[1] Great variability regarding microbiota composition and bifidobacteria levels can be found among different individuals, which could be affected by food intake, age, and individual characteristics, among other factors. Some health-promoting properties have been attributed to bifidobacteria, which are being included in a huge variety of fermented milks and functional foods.[2] Once ingested with food, probiotics must overcome biological barriers present in the gastrointestinal tract (GIT), mainly acid in the stomach and bile in the intestine. Several strategies are currently available to improve bifidobacterial survival, one of the more suitable being the pre-exposure to sublethal stresses or the selection of derivative strains by stress adaptation.[3] In this way, bifidobacteria can develop a stable bile-resistant phenotype; this adaptation process has been related to cross-resistance to other stressing factors as well as with other pleiotropic changes in cells.[4-7]

Many strains of lactic acid bacteria (LAB) and bifidobacteria are able to produce exopolysaccharides (EPS). Some EPS-producing strains are being used in the dairy industry because of their suitable technological properties.[8,9] However, the physiological functions of these biopolymers have not been clearly determined yet.[10] Among the beneficial effects attributed to EPS are a cholesterol-lowering ability, an immunomodulating capability, and the possibility of acting as prebiotics. Regarding their structure and composition, EPS from LAB are generally divided into homopolysaccharides (HoPS), which are polymers composed of one type of monosaccharide, and heteropolysaccharides (HePS), which are polymers of repeating

units that are composed of two or more types of monosaccharides. Studies on the physical properties, composition and structure of polymers produced by bifidobacteria are currently scarce.[10] We have recently found that some human intestinal *Bifidobacterium* isolates were able to produce HePS[11] that served as fermentable substrates for the human intestinal microbiota.[12] In addition, bile exposure is able to trigger the synthesis of EPS in *Bifidobacterium animalis.*[13] Acquisition of a bile-resistant phenotype has been recently associated with changes in the EPS fractions of some LAB and *Bifidobacterium* species of non-human origin. In this respect, the spontaneous acquisition of a "ropy" phenotype in a bile-resistant derivative of *B. animalis* was correlated with variations in molar mass (MM) and ratios among the monosaccharides glucose, galactose and rhamnose, which are components of the EPS fractions synthesized by original and bile-adapted strains.[14] In *Lactobacillus delbrueckii* subsp. *lactis*, the acquisition of a bile-resistant phenotype promoted slight changes in glycosidic linkages of the EPS which affected some technological and functional properties of the producing microorganism.[15]

Taking into consideration the data commented on above, the aim of the present work was to gain insight into the influence that adaptation to bile could exert on some physico-chemical and biological characteristics of EPS produced by the species *Bifidobacterium longum*, a normal inhabitant of the human GIT. The strain *B. longum* NB667 and its corresponding cholate-resistant derivative *B. longum* IPLA B667dCo were used as the model of study.

MATERIAL AND METHODS

EPS-producing strains and culture conditions

Two *B. longum* strains were used in this study. *B. longum* NB667 from NIZO Food Research Collection (Ede, the Netherlands) was originally isolated from infant feces. *B. longum* IPLA B667dCo is a cholate-adapted strain, belonging to the IPLA Collection, and obtained from *B. longum* NB667 in a previous work.[5] The strains were maintained at -80 ºC in MRSC broth [MRS broth (Biokar Diagnostics, Beauvais, France) plus 0.25% (w/v) L-cysteine (Sigma-Adrich Chemical Co., St. Louis, MO, USA)] with 20% glycerol. For the isolation of EPS, frozen stocks were grown overnight in MRSC at 37 ºC in an anaerobic cabinet (Mac 500; Don Whitley Scientific, West Yorkshire, UK) under a 10% H_2, 10% CO_2, and 80% N_2 atmosphere. Cultures (200 μL) were used to inoculate agar-MRSC plates by means of sterile glass beads. Plates were incubated for 5 days at 37 ºC in anaerobic conditions.

EPS isolation

Crude EPS produced by *Bifidobacterium* strains were purified from the cellular biomass harvested from MRSC agar plates.[16] Biomass was collected using 2 mL ultrapure water per plate and the resulting volume mixed with 1 volume of 2 M NaOH. The suspension was gently stirred overnight at room temperature to promote polymer release from the cellular surface. Cells were removed by centrifugation at 8,400 x *g* for 30 min and EPS was precipitated from the supernatants over 48 h at 4 ºC using 2 volumes of cold absolute ethanol. Following centrifugation, the EPS fraction was resuspended in ultrapure water and dialyzed for 3 days at 4 ºC with daily changes of water, using dialysis tubes (Sigma) of 12 to 14 kDa molecular mass cut off. Finally, the dialyzed crude EPS preparations were freeze-dried.

Fractionation of the EPS by size exclusion chromatography (SEC)

For analytical purposes, the weight average MM (Mw) distribution of the crude EPS was measured by SEC and multi-angle laser light scattering detection (SEC-MALLS) as described by Salazar et al.[17] Diffusion ordered NMR spectroscopy (DOSY) was also used as an additional procedure to ascertain the Mw of these polymers through the measurement of diffusion coefficients[18] as described by Leal et al.[19] The protein content of the polymers was determined by the BCA protein assay kit (Pierce, Rockford, IL, USA) following the manufacturer´s instructions.

For EPS fractionation preparative SEC was used: 20 mg of crude EPS were dissolved in 2 mL of 0.3 M NaOH and centrifuged at 16,000 x *g* for 15 min in an Eppendorf 5415D microcentrifuge to eliminate insoluble material. The supernatant was then subjected to SEC in a Sepharose CL6B (GE Healthcare, Madrid, Spain) column (60 x 2.6 cm) equilibrated with 0.3 M NaOH at a flow rate of 18 mL h^{-1}. EPS fractions (3.5 mL) were recovered and monitored for carbohydrates using the phenol-sulfuric acid method.[20] Apparent average MM of EPS eluted peaks was obtained using a calibration curve made with commercial standards (Dextran Blue, Dextrans T70, and T10 [Pharmacia, Uppsala, Sweden], and vitamin B12 [Merck, Darmdstadt, Germany]). The positive fractions for carbohydrates were appropriately combined, dialyzed against water, concentrated to a small volume, and freeze-dried. The amount of each purified fraction was determined gravimetrically.

Chemical characterization of EPS fractions

Monosaccharide composition and phosphate content

For analysis of neutral sugars in the two crude EPS and the SEC-fractionated EPS peaks, polysaccharides were hydrolyzed with 1.5 M trifluoraoacetic acid (TFA) for 1 h at 121 ºC. The resulting monosaccharides were converted into their corresponding

144 alditol acetates,[21] which were identified and quantified by gas-liquid chromatography
145 (GLC) on a 7980A instrument (Agilent Technologies Inc., Palo Alto, CA) equipped
146 with a flame ionization detector, using an Agilent HP5 fused silica column (30 m x 0.25
147 mm internal diameter x 0.2 μm film thickness) and the following temperature program:
148 160 °C for 5 min, then 3.5 °C min^{-1} to 205 °C and finally 210 ºC for 0.5 min. Phosphate
149 content was deduced from inorganic phosphate determination on a 5500 Inductively
150 Coupled Plasma instrument (Perkin Elmer, San Jose, CA, USA).
151
152 ***Monosaccharide linkage types***
153 The linkage types present in the EPS molecules were determined after
154 methylation of the SEC-fractioned EPS according to the procedure described by
155 Ciucanu and Kerek.[22] The permethylated polysaccharide was hydrolyzed with 3 M
156 TFA, and the released monosaccharides were reduced with $NaBD_4$ and then acetylated
157 to give their corresponding partially methylated alditol acetates, which were analyzed
158 by GLC and mass spectrometry (GLC-MS) under conditions previously described.[23]
159
160 ***Fourier-transform infrared spectroscopy (FT-IR)***
161 Fourier transform (FT) Infrared (IR) spectra were obtained by the KBr
162 technique[24] using a FTIR 4200 type A instrument (Jasco Corporation, Tokyo, Japan).
163 Transmittance was measured in a spectral range of 400–4000 cm^{-1}. A Deuterated L-
164 Alanine Triglycine Sulphate (TGS) detector was used with 4 cm^{-1} data resolution.
165
166 **Fermentation of bacterial EPS in fecal cultures**
167 ***Fecal batch cultures***
168 Three independent fecal batch fermentations, each of them corresponding to

169 samples of three different healthy adult donors (2 women and 1 man, 25 to 37 years
170 old), were carried out in the carbohydrate-free basal medium (CFBM) previously
171 described.[12] For each batch, CFBM was distributed into different glass tubes that were
172 added with the crude EPS isolated from each of the two *Bifidobacterium* strains. One
173 additional tube was kept without adding carbon source and was used as a control. The
174 fecal batch cultures were performed in the conditions indicated previously.[12] Samples
175 for analyses were taken from fecal cultures at times 0, 1, and 5 days. Eight hundred μL
176 of cultures were centrifuged (12000 x *g*, 10 min) each time, and pellets and supernatants
177 were collected.
178
179 ***Analysis of Short Chain Fatty Acids (SCFA) by GC-MS***
180 Cell-free supernatants from fecal batch cultures were filtered through 0.2 μm
181 filters and mixed with 1/10 of ethyl-butyric (2 mg mL^{-1}) as an internal standard and
182 stored at -20 ºC until analysis.
183 A system composed of a gas chromatograph (GC) 6890N (Agilent) connected
184 to a MS 5973N detector (Agilent) was used to quantify the SCFA. Data were collected
185 with the Enhanced ChemStation G1701DA software (Agilent). Samples (1 μL) were
186 analyzed as previously described by Salazar and co-workers.[12] Total SCFA
187 concentrations were calculated as the sum of the three major SCFA (acetic + propionic
188 + butyric). The molar proportion of each SCFA was obtained as the concentration
189 percentage with respect to the total SCFA.
190
191 ***Analysis of bifidobacteria by quantitative real-time PCR* (qPCR)**
192 The quantification of the *Bifidobacterium* population in fecal batch cultures
193 was performed by qPCR using previously described genus-specific primers.[25] DNA was

194 extracted from pellets harvested from 800 μL of fecal batch cultures. Cells were washed
195 once in PBS buffer and DNA was extracted with the QIAamp® DNA Stool Kit (Qiagen
196 GmbH, Hilden Germany) following the manufacturer's instructions. Purified DNA
197 samples were stored at -20 ºC until use.

198 All reactions were performed on MicroAmp optical plates sealed with
199 MicroAmp optical caps (Applied Biosystems, Foster City, CA) and amplifications were
200 carried out in a 7500 Fast Real Time PCR System (Applied Biosystems) using the
201 SYBR Green PCR Master Mix (Applied Biosystems). One μL of purified DNA was
202 used as the template in the 25 μL PCR reaction. Thermal cycling consisted of an initial
203 cycle of 95 ºC for 10 min followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min.
204 Standard curves were made with the strain *B. longum* NCIMB8809 which was grown
205 overnight in MRSC under anaerobic conditions. Standard curves were obtained by
206 plotting the C_t values obtained for the standard culture as a linear function of the base-
207 10 logarithm of the initial number of cells in the culture determined by plate counting.
208 The number of *Bifidobacterium* cells in fecal samples was determined by comparing the
209 C_t values obtained to the standard curve. The detection limit with primers and qPCR
210 conditions used was 5×10^4 cells g^{-1}. Samples were analyzed in duplicate in at least two
211 independent PCR runs.
212
213 ***Statistical analysis***

214 The statistical analysis of SCFA data was performed with the SPSS-PC 15.0
215 software package (SPSS Inc., Chicago, IL, USA). Independent one-way ANOVA tests
216 were performed at 1 and 5 days of incubation. Differences among culture conditions
217 (control, and EPS NB667 and IPLA B667dCo as carbon sources) were assessed by
218 means of the LSD (least significant difference) mean comparison test ($p < 0.05$).

RESULTS AND DISCUSSION

Physico-chemical characterization of the EPS from the parental and the cholate-resistant derivative strains

Crude EPS polymer preparations were isolated from strains grown on MRSC plates instead of a complex liquid medium in order to avoid contamination with glucomannans present in the yeast extract added to MRS medium, as previously indicated by Salazar et al.[12] The level of EPS production by *B. longum* NB667 and *B. longum* IPLA B667dCo was about 180-190 mg polymer per 50 plates in both microorganisms, similar to that reported previously for the parental strain NB667.[11] The content of protein in both crude EPS ranged between 2.2 and 2.7%, these values being in the same order as those previously reported by Salazar et al.[12] for EPS synthesized by bifidobacteria from intestinal origin.

With the aim of knowing the physico-chemical characteristics of the EPS synthesized by *B. longum* and the possible influence that the acquisition of resistance to cholate could exert on them, EPS crude preparations from the two microorganisms under study were first compared. Analysis by SEC confirmed that the EPS crude fractions of both strains contained two polymer peaks of low (around $4x10^3$ g mol^{-1}) and high (around 4 to $5x10^6$ g mol^{-1}) apparent average MM (Table 1), as was recently reported for the parental strain *B. longum* NB667.[14] In EPS NB667 both peaks were present in similar amounts, whereas in EPS IPLA B667dCo the biggest peak was slightly less abundant than the smaller one. The presence of two peaks of different MM seems to be a relatively common feature in EPS fractions isolated from bifidobacteria.[14,26-28] Analysis by SEC-MALLS of crude EPS preparations revealed clear differences on the physico-chemical

parameters (Mw, Rg, and ν) between peaks of low and high MM in both strains (Table 1). In addition, a significant amount of protein appeared associated with the peak of low MM (peak 2) in the two strains under study (Figure 1). Remarkably, Mw of peak 1 from EPS NB667 and IPLA B667dCo estimated by SEC-MALLS dropped dramatically with respect to the apparent MM calculated by retention times in SEC (Table 1) but were of the same order of magnitude as those obtained by DOSY (2.2×10^5 g mol^{-1} and 5.5×10^5 g mol^{-1} for peak 1 of EPS NB667 and IPLA B667dCo, respectively). Reasons for these discrepancies will be discussed later. Rhamnose, galactose and glucose were released after acid hydrolysis of the crude EPS preparations of both strains and minor amounts of mannose were also obtained; the monosaccharide relative ratios displayed minor differences between the parental strain and the cholate-resistant derivative (Table 1). Monosaccharide composition of both EPS crude polymers was similar to that found in other LAB and bifidobacteria from food and intestinal origin.[27, 29] The presence of significant amounts of uronic acids or amino sugars was ruled out in crude EPS preparations of both strains based on the lack of characteristic absorption bands in the IR-FT spectra (data not shown). Phosphate determinations rendered only trace amounts (0.6-1.0%).

Therefore, in a step forward crude EPS were submitted to preparative SEC in order to separate the two fractions of low and high MM for accomplishing further characterization of these two polymers. The separation of both fractions was complete (Figure 2); however, the monosaccharide recovery after acid hydrolysis of the bigger MM peak was low (about 40%) whereas that of the smaller peak was close to 80%. Other authors have also reported low yield of recovered monosaccharides from polymer fractions in the genus *Bifidobacterium.*[26, 33] Quantitative monosaccharide composition and methylation (monosaccharide linkage) analyses indicated that the EPS fraction of low MM (peak 2) produced by both bifidobacteria was an HePS mainly composed of rhamnose,

galactose and glucose (Table 1). Traces of mannose, xylose, N-acetyl-glucosamine and glucuronic acid were also found (data not shown). FT-IR spectra of these HePS had similar patterns and showed absorption bands characteristic of neutral polysaccharides (data not shown). Thus, from our results it is not clear at this point whether the proteins that appeared associated with the peak of low MM (peak 2) detected by SEC-MALLS were cellular contaminants or take part of the structure of the polymer; in any case, slight differences in monosaccharide proportions and linkage types were found between polymers of peak 2 from parental and derivative strains. Thus, a slightly lower proportion of glucose and galactose was obtained in the polymer of *B. longum* IPLA B667dCo as compared to the polymer of the parental strain (Table 1); this feature was consistent with the lower proportion of residues →4)-Glc*p*-(1→ and →3)-Gal*p*-(1→ in the EPS fraction of the derivative strain (Table 2). Relating to this, we have recently reported variations in MM and monosaccharide ratios between polymers synthesized by a bile resistant *B. animalis* and its parental strain[14] as well as slight variations in monosaccharide linkage proportions between EPS produced by a *L. delbrueckii* subsp. *lactis* strain and its bile resistant derivative.[15] All these findings suggest that adaptation to bile could promote changes in the composition and physico-chemical characteristics of EPS.

The carbohydrate content of the high MM peak (peak 1) was similar in EPS from both *B. longum* NB667 and IPLA B667dCo strains (Table 1). It consisted of a HePS formed by galactose and glucose in molar ratios of 1:1.8, which presented similar proportions of sugar linkage types in both microorganisms (Table 2). Traces of mannose were also found. To have a more precise characterization of this high MM fraction and to determine possible differences involving non-carbohydrate molecules, the FT-IR spectra in the region 3,750-400 cm^{-1} was obtained and analyzed (Figure 3). The intensity of bands around 3,400, 1,400, and 1,060 cm^{-1} is due to the hydroxyl stretching vibration of the

294 polysaccharides, and was similar in the peaks of both polymers. The absorption band at
295 2,927 cm^{-1} corresponds to methyl groups and it was also present in this polymer fraction of
296 both microorganisms; since no monosaccharides containing methyl groups were found in
297 this peak, the presence of this FT-IR band may be associated to other non-carbohydrate
298 structures taking part of the polymer. The region between 1,860 and 1,660 cm^{-1} revealed
299 the presence of carbonyl groups. In this region, some differences were evidenced between
300 the high MM EPS fractions synthesized by the parental and the cholate-resistant strains.
301 Thus, in EPS NB667 a weak absorption band appeared at 1,727 cm^{-1} whereas a
302 considerably more intense band appeared at 1,685 cm^{-1} in the EPS fraction of the cholate-
303 resistant strain IPLA B667dCo. Some differences were also found at 1,209 and 1,131 cm^{-1}
304 between fractions of both strains. Moreover, DOSY spectra also corroborated a higher
305 heterogeneity in the peak 1 from EPS IPLA B667dCo than in the peak 1 from EPS NB667
306 (data not shown). These results indicated that the fractions of high MM found in EPS
307 polymers from the strains *B. longum* NB667 and IPLA B667dCo were equal in
308 monosaccharide content, but presented some differences in the infrared spectra regarding
309 compounds other than carbohydrates that could also be associated to the polymers. The
310 presence of these non-carbohydrate structures may be the reason for the low
311 monosaccharide recovery obtained after the acid hydrolysis of the high MM fraction; this
312 fact and mismatches between the hydrodynamic volume and the true Mw may be
313 contributing to the discrepancies observed between the apparent MM and Mw obtained for
314 peak 1 using different techniques. The elucidation of the chemical nature of these non-
315 carbohydrate residues and the structure of the high MM fraction present in the EPS
316 synthesized by the parental and the cholate derivative *B. longum* strains should take
317 advantage from using nuclear magnetic resonance spectroscopy and mass spectrometry
318 techniques and constitutes a research challenge for the near future. Thus, it seems that the

adaptation to bile salts could not only induce the synthesis of EPS [13], but also produce modifications in the physico-chemical characteristics of the synthesized polymers.

EPS and cell wall polysaccharides described so far in the species *B. longum* are HePS, which seems to be a common feature in the genus *Bifidobacterium.*[26-28, 30-33] Galactose is present in all polymers of the species *B. longum* currently known, being the major component of most of them, [26, 28, 30-32] as it was also the case of the EPS produced by our strains *B. longum* NB667 and IPLA B667dCo. Indeed, in the peak of low MM galactose was the most abundant monosaccharide.

Fermentation of crude EPS fractions by the human intestinal microbiota

The bifidogenic effect of EPS purified from intestinal bifidobacteria isolated from healthy adults, as well as their ability to be fermented by the human intestinal microbiota has recently been demonstrated in our group. [12, 17] In the same way, we have used in the current work the crude EPS polymers isolated from the strain NB667 from infant origin, and from the strain IPLA B667dCo, adapted to cholate, in order to know if they can also be fermented by the intestinal microbiota and whether the physico-chemical changes promoted by the acquisition of resistance to cholate could modify the general fermentation pattern of *B. longum* EPS preparations.

The increase of total SCFA after 5 days of incubation in fecal cultures was significantly ($P < 0.05$) more pronounced in the presence of EPS than in the control cultures without any carbon source added, indicating that the EPS from *B. longum* were fermented by the intestinal bacteria (Table 3). Acetic acid was the most abundant SCFA, followed by propionic and butyric acids. Molar proportions of acetic and propionic acids decreased and increased, respectively, during incubation in the presence

of EPS preparations of both strains, whereas proportions of butyric acid did not suffer noticeable variations. This fermentation pattern coincided with that previously observed by us for other EPS preparations from *B. longum* strains of human intestinal origin.[12] As a consequence of the SCFA production pattern just indicated, the acetic acid to propionic acid ratio decreased during incubation in the presence of EPS from all microorganisms tested in the present work and in previous studies. Interestingly, cultures with EPS NB667 and IPLA B667dCo displayed slightly more pronounced decreases and lower final values for the acetic acid to propionic acid ratio than cultures with EPS from other bifidobacteria of adult intestinal origin previously tested by us, including several isolates of *B. longum* species.[12] In spite of this, no significant differences were evidenced in the present work on the levels of total SCFA, acetic, propionic and butyric acids formed during incubation between fecal cultures with EPS preparations of the strains *B. longum* NB667 and IPLA B667dCo (Table 3). The reduction in the acetic acid to propionic acid ratio has been proposed as a possible indicator of the hypolipidemic effect of prebiotics,[34] leading to a decrease of lipid levels in blood.

The high inter-individual difference among fecal cultures of the different donors precluded the statistical analysis; then, qPCR counts of fecal cultures of each individual were considered separately. A moderate stimulatory effect on intestinal *Bifidobacterium* populations as compared to the control (without carbohydrate added), was obtained for EPS crude preparations of both *B. longum* strains in the fecal cultures of only one individual (donor 3) out of the three analyzed (Figure 4). This behavior was similar to that previously reported with EPS from other bifidobacteria of adult intestinal origin.[12] No remarkable differences were found between the crude EPS preparations from the parental and the cholate-resistant strains, as it can be expected from minor

368 variations in the carbohydrate composition encountered between the polymers of both
369 microorganisms.
370
371 Several surface macromolecules of Gram-positive bacteria, among which are
372 glycosylated structures and EPS, could act as signal molecules interacting with eukaryotic
373 cells of the host. [35] Thus, variations in composition, as well as in proportions, between the
374 different monosaccharides and linkage types could modify the structural and functional
375 characteristics of the polymers and hence the possible influence in the cross-talk
376 mechanisms of the producing bacteria with the host. In this way, we have reported a
377 notable increase in hydrophobicity as well as enhanced *in vitro* adhesion to human
378 intestinal mucus of the cholate-resistant strain *B. longum* IPLA B667dCo with respect to
379 the parental sensitive strain *B. longum* NB667.[36]
380 In the current work we have corroborated, by using two strains of the species
381 *B. longum* as a model of study, that the acquisition of bile salt resistance promoted
382 changes in the physico-chemical characteristics of EPS. Thus, we suggest that these
383 modifications in EPS production may be a mechanism of response to bile stress
384 although this hypothesis, as well as the ecological relevance of these changes, deserves
385 future confirmation. On the other hand, differences in composition and glycosidic
386 linkages of the polymer fractions produced by parental and cholate-resistant strains
387 were not affecting their capability to be fermented by the intestinal microbiota. To the
388 best of our knowledge this is the first report on changes promoted by the acquisition of
389 a stable bile-resistance phenotype in the characteristics of EPS produced by
390 microorganisms of the species *B. longum*.
391

LITERATURE CITED:

(1) Harmsen, H.J.; Wideboer-Veloo, A.C; Raangs, G.C.; Wagendorp, A.A.; Klijn, N.; Bindels, J.G.; Welling, G.W. Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. *J. Pediatr. Gastroenterol. Nutr.* **2000**, *30***,** 61-67.

(2) Masco, L.; Huys, G.; de Brandt, E.; Temmerman, R.; Swings, J. Culture-dependent and culture-independent qualitative analysis of probiotic products claimed to contain bifidobacteria. *Int. J. Food Microbiol.* **2005**, *102***,** 221–230.

(3) Ruiz, L.; Ruas-Madiedo, P.; Gueimonde, M.; de los Reyes-Gavilán, C.G.; Margolles, A.; Sánchez, B. How do bifidobacteria counteract environmental challenges? Mechanisms involved and physiological consequences. *Genes Nutr.* **2011**, *6*, 307-318.

(4) Gueimonde, M.; Margolles, A.; de los Reyes-Gavilán, C.G.; Salminen, S. Competitive exclusion of enteropathogens from human intestinal mucus by *Bifidobacterium* strains with acquired resistance to bile-a preliminary study. *Int. J. Food Microbiol.* **2007**, *113*, 228-232.

(5) Noriega, L.; Gueimonde, M.; Sánchez, B.; Margolles, A.; de los Reyes-Gavilán, C.G. Effect of the adaptation to high bile salts concentrations on glycosidic activity, survival at low pH and cross-resistance to bile salts in *Bifidobacterium. Int. J. Food Microbiol.* **2004**, *94*, 79-86.

(6) Noriega, L.; de los Reyes-Gavilán, C.G.; Margolles, A. Acquisition of bile salt resistance promotes antibiotic susceptibility changes in *Bifidobacterium*. *J. Food Prot.* **2005**, *68*, 1916-1919.

(7) Ruas-Madiedo, P.; Hernández-Barranco, A.M.; Margolles, A.; de los Reyes-Gavilán, C.G. A bile salt-resistant derivative of *Bifidobacterium animalis* has an altered fermentation pattern when grown on glucose and maltose. *Appl. Environ. Microbiol.* **2005**, *71,* 6564-6570.

(8) Abraham, A.G.; Medrano, M.; Piermaria, J.A.; Mozzi, F. Novel applications of polysaccharides from lactic acid bacteria: a focus on kefiran. In *Food Hydrocolloids: characteristics, properties and structures*. Hollingworth, C.S. Ed.; Nova Publishers: New York, 2010; 253-271.

(9) Behare, P.V.; Rameshwar, S.; Kumar, M.; Prajapati, J.B.; Singh, R.P. Exopolysaccharides of lactic acid bacteria: a review. *J. Food Sci. Technol.* **2009**, *46*, 1-11.

(10) Ruas-Madiedo, P.; Salazar, N.; de los Reyes-Gavilan, C.G. Functionality of exopolysaccharides produced by lactic acid bacteria. In *Molecular aspects of lactic acid bacteria for traditional and new applications.* Mayo, B.; López, P.; Pérez-Martínez, G., Eds.; Research Signpost: Trivandrum, Kerala, India, 2008; 137–166.

(11) Ruas-Madiedo, P.; Moreno, J.A.; Salazar, N.; Delgado, S.; Mayo, B.; Margolles, A.; de los Reyes-Gavilán, C.G. Screening of exopolysaccharide-producing *Lactobacillus* and *Bifidobacterium* strains isolated from the human intestinal microbiota. *Appl. Environ. Microbiol.* **2007**, *73,* 4385–4388.

(12) Salazar, N.; Gueimonde, M.; Hernández-Barranco, A.M.; Ruas-Madiedo, P.; de los Reyes-Gavilán, C.G. Exopolysaccharides produced by intestinal *Bifidobacterium* strains act as fermentable substrates for human intestinal bacteria. *Appl. Environ. Microbiol*. **2008**, *74*, 4737-4745.

(13) Ruas-Madiedo, P.; Gueimonde, M.; Arigoni, F.; de los Reyes-Gavilán,

C.G.; Margolles, A. Bile affects the synthesis of exopolysaccharides by *Bifidobacterium animalis*. *Appl. Environ. Microbiol.* **2009**, *75,* 1204–1207.

(14) Ruas-Madiedo, P.; Medrano, M.; Salazar, N.; de los Reyes-Gavilán, C.G.; Pérez, P.F.; Abraham, A.G. Exopolysaccharides produced by *Lactobacillus* and *Bifidobacterium* strains abrogate *in vitro* the cytotoxic effect of bacterial toxins on eukaryotic cells. *J. Appl. Microbiol.* **2010**, *109*, 2079-2086.

(15) Burns, P.; Vinderola, G.; Reinheimer, J.; Cuesta, I.; de los Reyes-Gavilán, C.G.; Ruas-Madiedo. P. Technological characterization and survival of the exopolysaccharide-producing strain *Lactobacillus delbrueckii* subsp. *lactis* 193 and its bile-resistant derivative 193+ in simulated gastric and intestinal juices. *J. Dairy Res.* **2011,** *78*, 357-364.

(16) Ruas-Madiedo, P.; Gueimonde, M.; Margolles, A.; de los Reyes-Gavilán, C.G.; Salminen, S. Exopolysaccharides produced by probiotic strains modify the adhesion of probiotics and enteropathogens to human intestinal mucus. *J. Food Prot*. **2006**, *69***,** 2011-2015.

(17) Salazar, N.; Ruas-Madiedo, P.; Kolida, S.; Collins, M.; Rastall, R.; Gibson, G.; de los Reyes-Gavilán, C.G. Exopolysaccharides produced by *Bifidobacterium longum* IPLA E44 and *Bifidobacterium animalis* subsp. *lactis* IPLA R1 modify the composition and metabolic activity of human faecal microbiota in pH-controlled batch cultures. *Int. J. Food Microbiol*. **2009**, *135*, 260-267.

(18) Stilbs, P. Molecular self-diffusion coefficients in Fourier transform nuclear magnetic resonance spectrometric analysis of complex mixtures. *Anal. Chem.* **1981,** *53***,** 2135-2137.

(19) Leal, J.A.; Giménez-Abián, M.I.; Canales, Á.; Jiménez-Barbero, J.; Bernabé, M.; Prieto, A. Cell wall polysaccharides isolated from the fungus *Neotestudina rosatii*, one of the etiologic agents of mycetoma in man. *Glycoconj. J.* **2009,** *26,* 1047-1054.

(20) Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.; Smith, F. Colorimetric method for the determination of sugar and related substances. *Anal. Chem*. **1956**, *28*, 350-356.

(21) Laine, R.A.; Esselman, W.J.; Sweeley, C.C. Gas-liquid chromatography of carbohydrates. *Meth. Enzymol.* **1972**, *28***,** 159-167.

(22) Ciucanu, I.; Kerek, F. A simple and rapid method for the permethylation of carbohydrates. *Carbohydr. Res.* **1984**, *13***,** 209-217.

(23) Leal, J.A.; Jiménez-Barbero, J.; Bernabé, M.; Prieto, A. Structural elucidation of a cell wall fungal polysaccharide isolated from *Ustilaginoidea virens*, a pathogenic fungus of *Oriza sativa* and *Zea mays*. *Carbohydr. Res.* **2008**, *343*, 2980-2984.

(24) Price, W.J. Sample handling techniques, In *Laboratory methods in infrared spectroscopy*. R.G.J. Miller, R.G.J.; Stacc, B.C., Eds.; Heyden and Son: London, England, 1972; 97-128.

(25) Gueimonde, M.; Tölkko, S.; Korpimäki, T.; Salminen, S. New real-time quantitative PCR procedure for quantification of bifidobacteria in human fecal samples. *Appl. Environ. Microbiol.* **2004**, *70*, 4165-4169.

(26) Abbad-Andaloussi, S.; Talbaoui, H.; Marczak, R.; Bonaly, R. Isolation and characterization of exocellular polysaccharides produced by *Bifidobacterium longum. Appl. Microbiol. Biotechnol***. 1995***, 43*, 995-1000.

(27) Salazar, N.; Prieto, A.; Leal, J.A.; Mayo, B.; Bada-Gancedo, J.C.; de los

490 Reyes-Gavilán, C.G.; Ruas-Madiedo, P. Production of exopolysaccharides by
491 *Lactobacillus* and *Bifidobacterium* strains from human origin and metabolic
492 activity of the producing bacteria in milk. *J. Dairy Sci.* **2009,** *92*, 4158–4168.
493 (28) Tone-Shimokawa, Y.; Toida, T.; Kawashima, T. Isolation and structural
494 analysis of polysaccharide containing galactofuranose from the cell walls of
495 *Bifidobacterium infantis*. *J. Bacteriol.* **1996**, *178*, 317-320.
496 (29) Mozzi, F.; Vaningelgem, F.; Hébert, E.M.; van der Meulen, R.; Foulquié-
497 Moreno, M.R.; Font de Valdéz, G.; De Vusyt, L. Diversity of
498 heteropolysaccharide-producing lactic acid bacterium strains and their
499 biopolymers. *Appl. Environ. Microbiol*. **2006**, *72*, 4431-4435.
500 (30) Nagaoka, M.; Shibata, H.; Kimura, L.; Hashimoto, S.; Kimura, K.;
501 Sawada, H.; Yokokura, T. Structural studies on a cell wall polysaccharide from
502 *Bifidobacterium longum* YIT4028. *Carbohydr. Res.* **1995**, *274***,** 245-249.
503 (31) Roberts, C.; Fett, W.F.; Osman, S.F.; Wikey, C.; O'Connor, J.V.;
504 Hoover, D.G. Exopolysaccharide production by *Bifidobacterium longum* BB-79.
505 *J. Appl. Bacteriol.* **1995**, *78*, 463-468.
506 (32) Kohno, M.; Suzuki, S.; Kanaya, T.; Yoshino, T.; Matsuura, Y.; Asada,
507 M.; Kitamura, S. Structural characterization of the extracellular polysaccharide
508 produced by *Bifidobacterium longum* JBL05. *Carbohydr. Polym.* **2009**, *77***,** 351-
509 357.
510 (33) Xu, R.; Shen, Q; Ding, X.; Gao, W; Li, P. Chemical characterization and
511 antioxidant activity of an exopolysaccharide fraction isolated from
512 *Bifidobacterium animalis* RH. *Eur. Food Res. Technol.* **2011**, *232*, 231-240.
513 (34) Delzenne, N.M.; Kok, N. Effects of fructan-type prebiotics on lipid
514 metabolism. *Am. J. Clin. Nutr.* **2001**, *73*, 456S-458S.

(35) Lebeer, S.; Vanderleyden, J.; de Keersmaecker, S.C.J. Host interactions of probiotic bacterial surface molecule: comparison with commensals and pathogens. *Nat. Rev. Microbiol.* **2010**, *8,* 171-184.

(36) Gueimonde, M.; Noriega, L.; Margolles, A.; de los Reyes-Gavilán, C.G.; Salminen, S. Ability of *Bifidobacterium* strains with acquired resistance to bile to adhere to human intestinal mucus. *Int. J. Food Microbiol.* **2005**, *101*, 341-346.

FUNDING SOURCES

This work was financially supported by the European Union FEDER funds and by the Plan Nacional de I + D under projects AGL2004-6088, AGL2007-62736, and AGL2010-16525. Nuria Salazar was the recipient of a predoctoral fellowship from the Spanish Ministry of Science and Innovation (FPI Program).

FIGURE CAPTIONS

Figure 1. Size exclusion chromatography (SEC) analysis of the EPS crude fractions produced by the strains *Bifidobacterium longum* NB667 and IPLA B667dCo. The EPS were dissolved in 0.1 M $NaNO_3$ (5 mg ml^{-1}) and separation was carried out in a HPLC system (Alliance 2690 module injector, Waters, Milford, MA, USA) using two TSK-Gel columns (Sigma-Supelco) placed in series (G3000 PW_{XL} + G5000 PW_{XL} protected with a TSK-Gel guard column) at 40°C using 0.1 M $NaNO_3$ as mobile phase at flow rate 0.45 ml min^{-1}. After separation, three detectors placed in series were used: the refraction index detector (Waters RI, blue line) was used for detection of different molecules, the photodiode array (Waters PDA, green line) set at 280 nm was used to identify the presence of proteins, and the multiangle laser light scattering (MALLS) detector Dawn Heleos II (Wyatt Europe GmbH, Dembach, Germany) having eighteen angles of detection (detector 11 = angle 90°, red line) was used to study the molar mass distribution of the EPS fractions. The ASTRA software (Wyatt Europe) was used to analyse the scattering data using the Random Coil adjust-model.

Figure 2. Preparative size exclusion chromatography (pre-SEC) of the EPS crude polymers synthesised by the strains *Bifidobacterium longum* NB667 and IPLA B667dCo. Carbohydrates in eluted fractions were measured by the phenol-sulfuric acid method. Arrows indicate fractions collected from each elution peak. EPS NB667 (peak 1: fractions 28-48, peak 2: fractions 62-84); EPS IPLA B667dCo (peak 1: fractions 30-44, peak 2: fractions 60-85).

Figure 3. Fourier-transform infrared spectra of the peak of high MM purified by preparative-SEC in Sepharose CL6B from crude EPS polymers of the strains

Bifidobacterium longum NB667 (upper line) and *B. longum* IPLA B667dCo (bottom line). % T= percentage of transmittance. The dotted rectangles indicate areas of the spectrum displaying differences between parental and cholate resistant strains.

Figure 4. Increase, with respect to time zero, of *Bifidobacterium* counts measured by qPCR in fecal slurry cultures from three human adult donors in the absence of external carbohydrate added (control, white bars) and using crude EPS isolated from the strains *Bifidobacterium longum* NB667 (gray bars) and *B. longum* IPLA B667dCo (black bars) as carbon sources after 1 day (a) and 5 days (b) of incubation. Initial *Bifidobacterium* counts were as follows: 9.64±0.10 log cells g^{-1} for donor 1, 10.65±0.12 log cells g^{-1} for donor 2, and 9.93±0.08 log cells g^{-1} for donor 3. Vertical lines indicate standard deviation.

Table 1. Physico-chemical characteristics of the crude EPS polymers produced by the strains *Bifidobacterium longum* NB667 and IPLA B667dCo. The weight average molar mass (M_w, g mol^{-1}), weight radius of gyration (R_w, nm) as well as the coefficient ν (log R_w/log M_w) were determined by SEC-MALLS (see Figure 1). The crude EPS polymers were fractionated by preparative SEC (see Figure 2) and the apparent average molar mass (MM) (g mol^{-1}) of the fractions (peaks) as well as their monosaccharide composition were determined by phenol-sulfuric method using commercial standards, and GC-FID, respectively. Glc: glucose, Gal: galactose, Rha: rhamnose.

	EPS NB667			**EPS IPLA B667dCo**		
	Crude EPS	Peak 1	Peak 2	Crude EPS	Peak 1	Peak 2
M_w	-	$5.5x10^5$	$7.2x10^3$	-	$7.8x10^5$	$7.6x10^3$
R_w (nm)	-	67.8	22.9	-	69.8	24.4
ν (log R_w/log M_w)	-	0.35	1.52	-	0.31	1.18
Apparent MM and distribution (%)	-	$4.3x10^6$ (49%)	$4.3x10^3$ (51%)	-	$5.4x10^6$ (41%)	$4.5x10^3$ (59%)
Monosaccharide ratio						
Glc	1.0	1.8	1.2	1.2	1.8	1.0
Gal	2.2	1.0	1.8	2.3	1.0	1.7
Rha	1.0	0.0	1.0	1.0	0.0	1.0

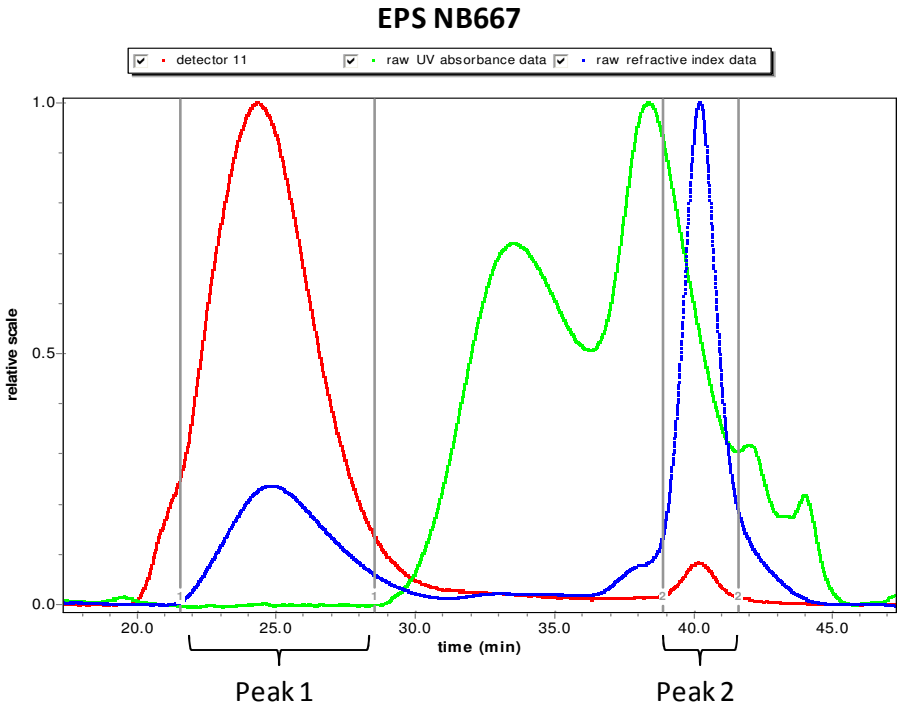
Table 2. Linkage types (ratios) deduced for EPS produced by the strains *Bifidobacterium longum* NB667 and *B. longum* IPLA-B667dCo. Glc: glucose, Gal: galactose, Rha: rhamnose, Hex: hexose, *p*: pyranose ring conformation, *f*: furanose ring conformation.

Linkage	EPS NB667		EPS IPLA-B667dCo	
	Peak 1	Peak 2	Peak 1	Peak 2
Glc*p*-(1→	0.5		0.5	
→2,4)-Glc*p*-(1→	1.0		1.0	
→4)-Gal*p*-(1→	1.0	0.2	1.0	0.2
→4)-Glc*p*-(1→	0.7	1.2	0.7	0.7
Hex*p*-(1→		0.1		0.3
Gal*p*-(1→		0.9		0.9
→2)-Rha*p*-(1→		1.0		1.0
→3)-Gal*f*-(1→		0.1		0.1
→3)-Gal*p*-(1→		0.7		0.6
→6)-Hex*p*-(1→		0.2		0.2
→3,6)-Gal*p*-(1→		1.0		1.0

Table 3. Molar concentrations and proportion of the three major SCFA in fecal cultures from three adult healthy donors without carbohydrate added (control) and using isolated crude EPS preparations from *Bifidobacterium longum* NB667 and *B. longum* IPLA-B667dCo as carbon sources. The differences among the three "carbon source" groups in each sampling point were tested by means of independent one-way ANOVA. The means that do not share a common superscript are significantly different ($p < 0.05$) accordingly to the mean comparison test LSD.

Time (days)	Carbon source	Concentration ± SD (mM)				Proportion ± SD (%)			
		Total acid	Acetate	Propionate	Butyrate	Acetate	Propionate	Butyrate	A/P ratio
0	Control	6.27±2.68	4.10±1.86	0.96±0.40	1.22±0.42	64.77±1.82	15.27±1.23	19.95±2.15	4.26±0.35
1	Control	13.72±1.13	8.42±0.47	2.27±0.26	3.03±0.55	61.44±1.93	16.59±1.71	21.97±2.15	3.73±0.41
	EPS NB667	14.95±4.02	9.12±2.48	2.80±0.78	3.03±0.88	61.08±3.14	18.74±2.63	20.18±0.69	3.31±0.59
	EPS IPLA-B667dCo	14.83±0.41	8.88±0.43	2.80±0.36	3.15±0.46	59.96± 4.15	18.87±2.24	21.17±2.54	3.22±0.61
5	Control	12.33±1.70^{a}	8.16±1.33^{a}	1.90±0.67^{a}	2.27±0.79	66.34±7.55	15.10±3.51^{a}	18.55±6.32	4.58±1.27^{b}
	EPS NB667	18.65±2.66^{b}	10.23±0.63^{b}	5.11±1.22^{b}	3.31±0.82	55.33±4.98	21.12±2.92^{b}	17.55±2.07	2.07±0.43^{a}
	EPS IPLA-B667dCo	20.71±4.17^{b}	11.16±1.26^{b}	5.82±1.72^{b}	3.72±1.24	54.60±5.58	27.74±3.20^{b}	17.65±2.47	2.00±0.46^{a}

Fig. 1


EPS NB667
detector 11
raw UV absorbance data
raw refractive index data
1.0
0.5
0.0
relative scale
20.0
25.0
30.0
35.0
40.0
45.0
time (min)
Peak 1
Peak 2

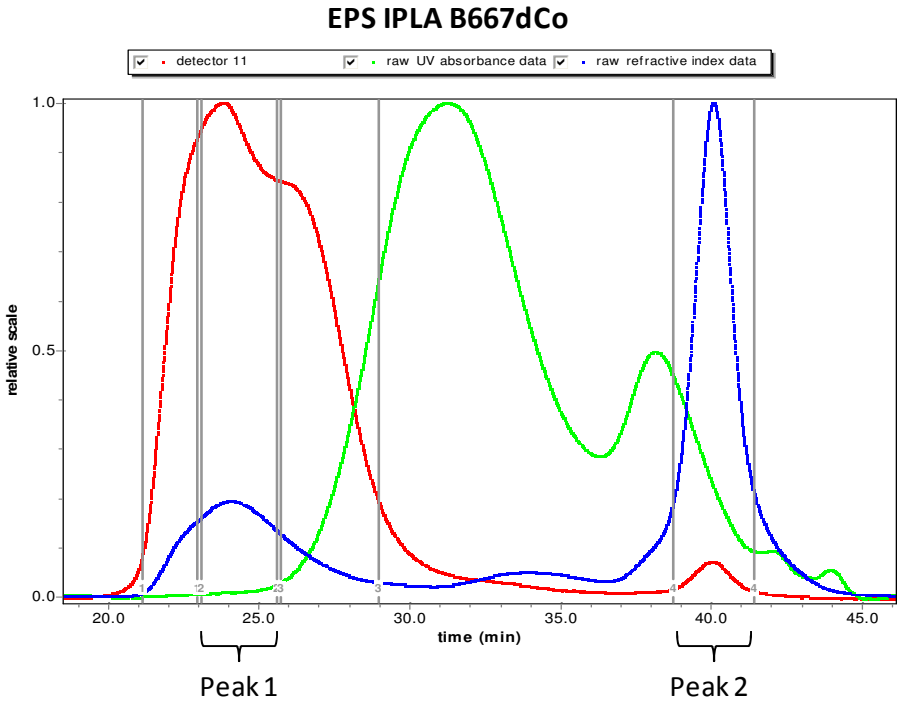

EPS IPLA B667dCo
detector 11
raw UV absorbance data
raw refractive index data
1.0
0.5
0.0
relative scale
20.0
25.0
30.0
35.0
40.0
45.0
time (min)
Peak 1
Peak 2

Fig. 2

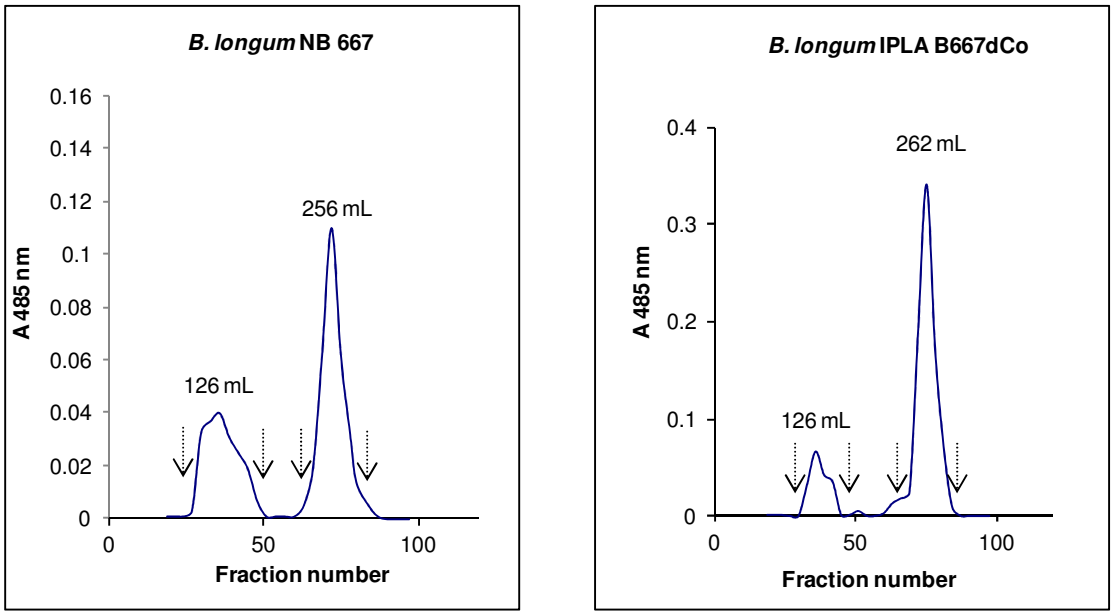

B. longum NB 667
A 485 nm
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
256 mL
126 mL
0
50
100
Fraction number
B. longum IPLA B667dCo
A 485 nm
0.4
0.3
0.2
0.1
0
262 mL
126 mL
0
50
100
Fraction number

Fig. 3

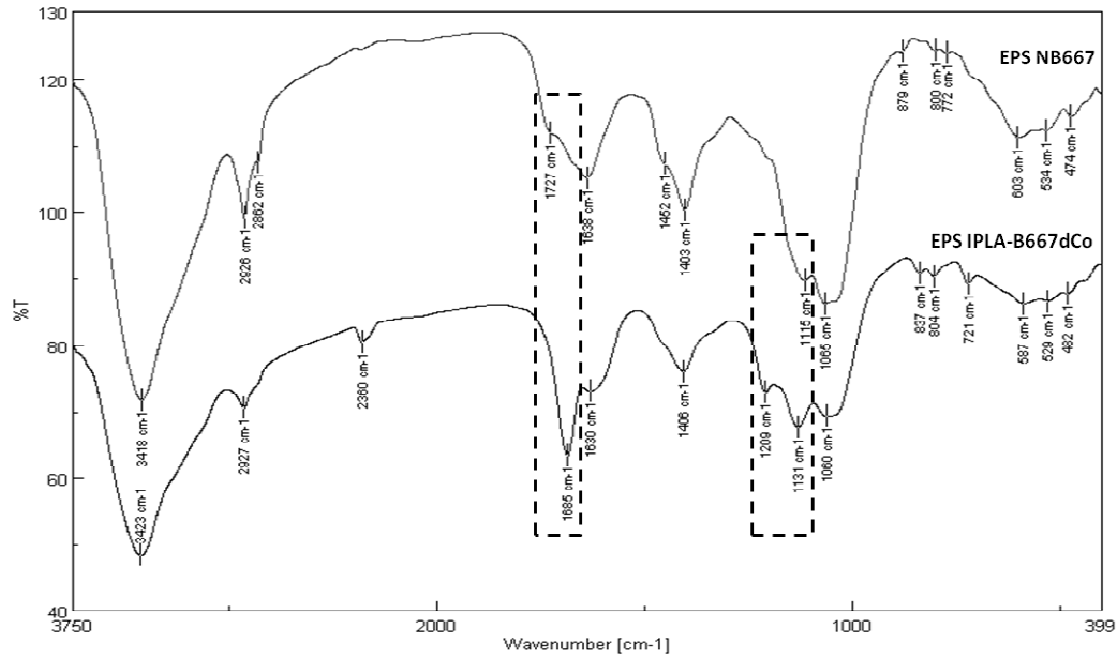

EPS NB667
EPS IPLA-B667dCo
%T
Wavenumber [cm-1]
130
120
100
80
60
40
3750
2000
1000
399
3418 cm-1
3423 cm-1
2926 cm-1
2892 cm-1
2927 cm-1
2360 cm-1
1721 cm-1
1638 cm-1
1685 cm-1
1630 cm-1
1452 cm-1
1403 cm-1
1406 cm-1
1209 cm-1
1131 cm-1
1115 cm-1
1085 cm-1
1060 cm-1
879 cm-1
800 cm-1
772 cm-1
837 cm-1
804 cm-1
721 cm-1
603 cm-1
534 cm-1
474 cm-1
587 cm-1
529 cm-1
482 cm-1

Fig. 4

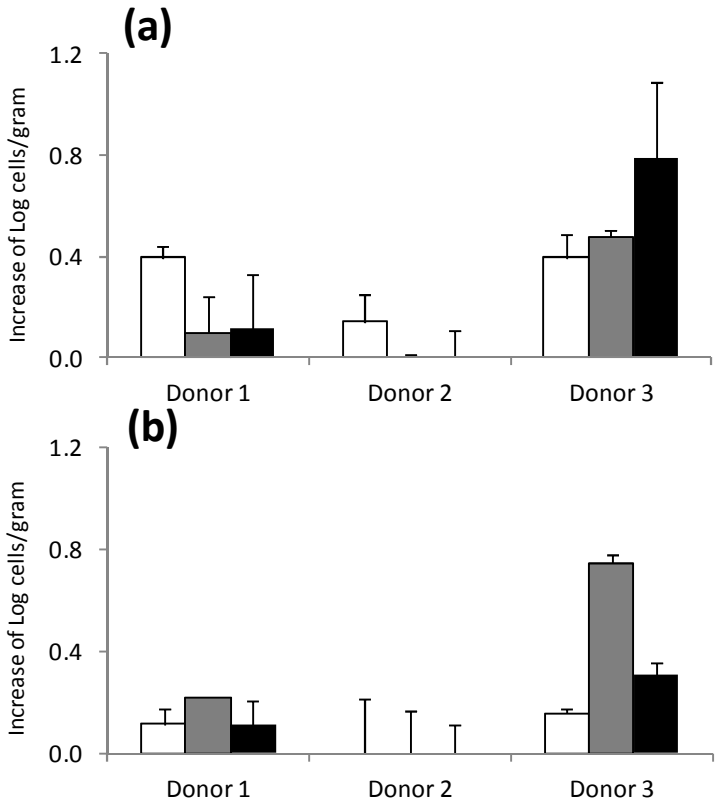

(a)
1.2
0.8
0.4
0.0
Increase of Log cells/gram
Donor 1
Donor 2
Donor 3
(b)
1.2
0.8
0.4
0.0
Increase of Log cells/gram
Donor 1
Donor 2
Donor 3

Figure for the Table of Contents