Characterization of Exopolysaccharides Produced by *Bifidobacterium longum* NB667 and Its Cholate-Resistant Derivative Strain IPLA B667dCo

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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. On the basis of 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 nonhuman 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 physicochemical 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.

■ MATERIALS 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 °C in MRSC broth [MRS broth (Biokar Diagnostics, Beauvais, France) plus 0.25% (w/v) L-cysteine (Sigma-Aldrich Chemical Co., St. Louis, MO, U.S.A.)] with 20% glycerol. For the isolation of EPS, frozen stocks were grown overnight in MRSC at 37 °C in an anaerobic cabinet (Mac 500; Don Whitley Scientific, West Yorkshire, U.K.) 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 °C 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 of 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 8400 × g for 30 min and EPS was precipitated from the supernatants over 48 h at 4 °C using 2 volumes of cold absolute ethanol. Following centrifugation, the EPS fraction was resuspended in ultrapure water and dialyzed for 3 days at 4 °C with daily changes of water, using dialysis tubes (Sigma) of 12−14 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 (M_w) distribution of the crude EPS was measured by SEC and multiangle 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 M_w 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, U.S.A.) 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 × 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 × 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, Darmstadt, 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 trifluoroacetic acid (TFA) for 1 h at 121 °C. The resulting monosaccharides were converted into their corresponding alditol acetates,[21] which were identified and quantified by gas−liquid chromatography (GLC) on a 7980A instrument (Agilent Technologies Inc., Palo Alto, CA) equipped with a flame ionization detector, using an Agilent HP5 fused silica column (30 m × 0.25 mm internal diameter × 0.2 μm film thickness) and the following temperature program: 160 °C for 5 min, then 3.5 °C min⁻¹ to 205 °C and finally 210 °C for 0.5 min. Phosphate content was deduced from inorganic phosphate determination on a 5500 Inductively Coupled Plasma instrument (Perkin-Elmer, San Jose, CA, U.S.A.).

Monosaccharide Linkage Types. The linkage types present in the EPS molecules were determined after methylation of the SEC-fractioned EPS according to the procedure described by Ciucanu and Kerek.[22] The permethylated polysaccharide was hydrolyzed with 3 M TFA, and the released monosaccharides were reduced with $NaBD_4$ and then acetylated to give their corresponding partially methylated alditol acetates, which were analyzed by GLC and mass spectrometry (GLC-MS) under conditions previously described.[23]

Fourier-Transform Infrared Spectroscopy (FT-IR). Fourier transform (FT) Infrared (IR) spectra were obtained by the KBr technique[24] using a FTIR 4200 type A instrument (Jasco Corporation, Tokyo, Japan). Transmittance was measured in a spectral range of 400−4000 cm⁻¹. A deuterated L-alanine triglycine sulfate (TGS) detector was used with 4 cm⁻¹ data resolution.

Fermentation of Bacterial EPS in Fecal Cultures. *Fecal Batch Cultures.* Three independent fecal batch fermentations, each of them corresponding to samples of three different healthy adult donors (2 women and 1 man, 25 to 37 years old), were carried out in the carbohydrate-free basal medium (CFBM) previously described.[12] For each batch, CFBM was distributed into different glass tubes that were added with the crude EPS isolated from each of the two *Bifidobacterium* strains. One additional tube was kept without adding carbon source and was used as a control. The fecal batch cultures were performed in the conditions indicated previously.[12] Samples for analyses were taken from fecal cultures at times 0, 1, and 5 days. Eight hundred microliters of cultures were centrifuged (12 000 × g, 10 min) each time, and pellets and supernatants were collected.

Analysis of Short Chain Fatty Acids (SCFA) by GC-MS. Cell-free supernatants from fecal batch cultures were filtered through 0.2 μm filters and mixed with 1/10 of ethyl-butyric (2 mg mL⁻¹) as an internal standard and stored at −20 °C until analysis.

A system composed of a gas chromatograph (GC) 6890N (Agilent) connected to a MS 5973N detector (Agilent) was used to quantify the SCFA. Data were collected with the Enhanced ChemStation G1701DA software (Agilent). Samples (1 μL) were analyzed as previously described by Salazar and co-workers.[12] Total SCFA concentrations were calculated as the sum of the three major SCFA (acetic + propionic + butyric).

Table 1. Physicochemical Characteristics of the Crude EPS Polymers Produced by the Strains *Bifidobacterium longum* NB667 and IPLA B667dCo[a]

	EPS NB667			EPS IPLA B667dCo		
	crude EPS	peak 1	peak 2	crude EPS	peak 1	peak 2
M_w		5.5×10^5	7.2×10^3		7.8×10^5	7.6×10^3
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 (%)		4.3×10^6	4.3×10^3		5.4×10^6	4.5×10^3
		(49%)	(51%)		(41%)	(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

[a]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.

The molar proportion of each SCFA was obtained as the concentration percentage with respect to the total SCFA.

Analysis of Bifidobacteria by Quantitative Real-Time PCR (qPCR). The quantification of the *Bifidobacterium* population in fecal batch cultures was performed by qPCR using previously described genus-specific primers.[25] DNA was extracted from pellets harvested from 800 μL of fecal batch cultures. Cells were washed once in PBS buffer and DNA was extracted with the QIAamp DNA Stool Kit (Qiagen GmbH, Hilden Germany) following the manufacturer's instructions. Purified DNA samples were stored at −20 °C until use.

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

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

■ RESULTS AND DISCUSSION

Physicochemical 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 of 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 physicochemical 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 4×10^3 g mol^{-1}) and high (around 4 to 5×10^6 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 physicochemical parameters (M_w, R_w, 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, M_w 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
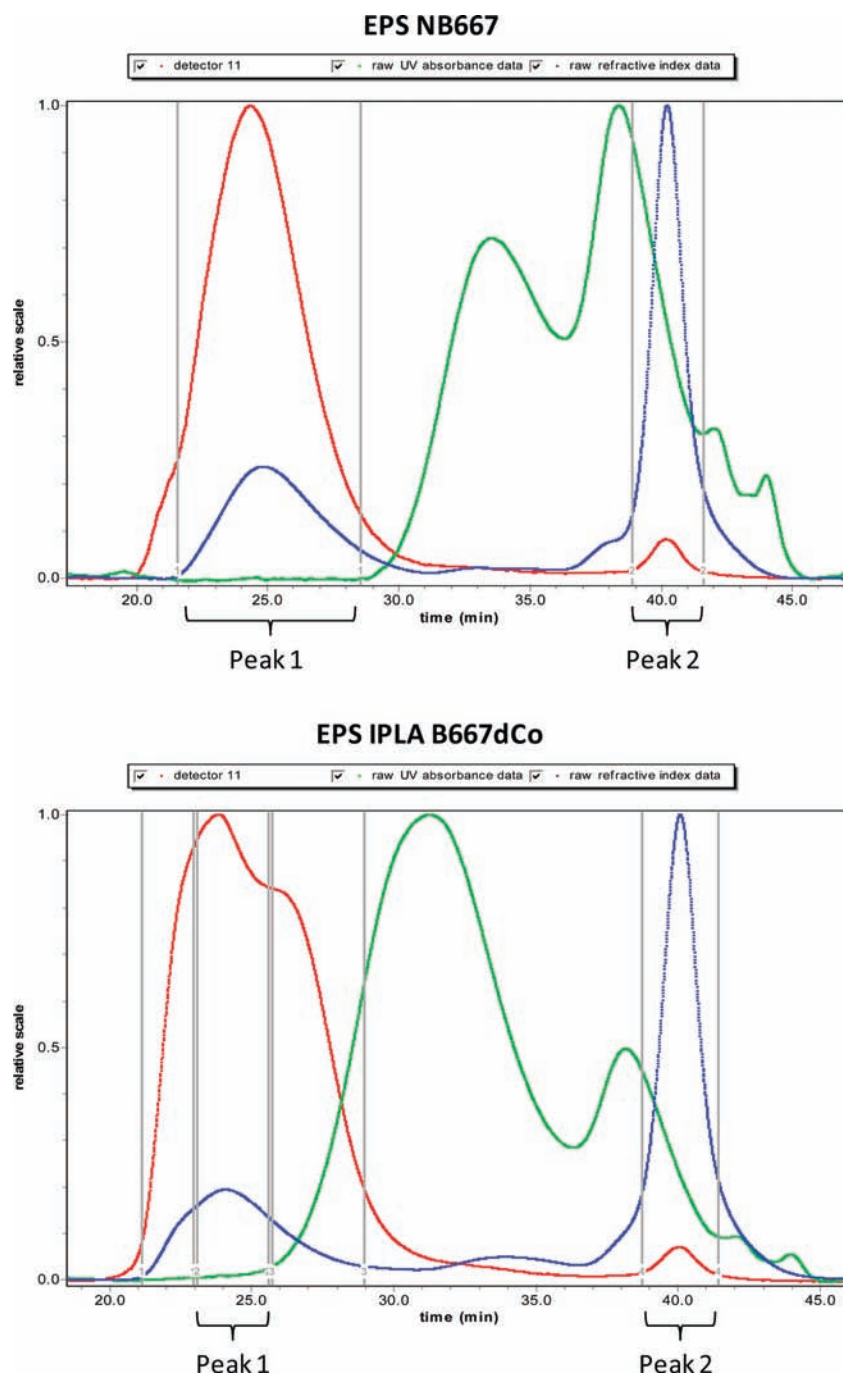
Figure 1. Size exclusion chromatography (SEC) analysis of the EPS crude fractions produced by the strains *Bifidobacterium longum* NB667 and IPLA B667dCo. The EPS were dissolved in 0.1 M NaNO$_3$ (5 mg mL^{-1}) and separation was carried out in a HPLC system (Alliance 2690 module injector, Waters, Milford, MA, U.S.A.) 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 analyze the scattering data using the random coil adjust-model.

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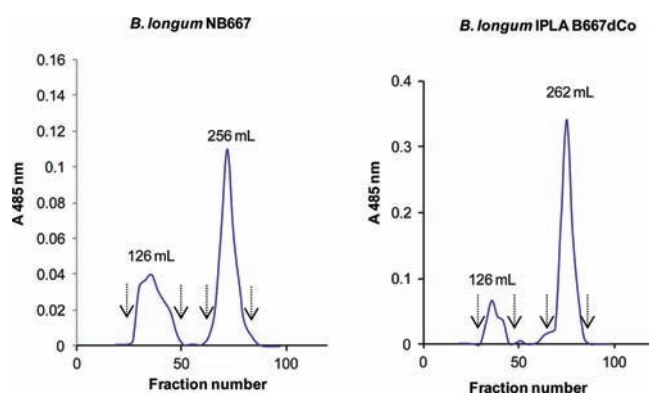
Figure 2. Preparative size exclusion chromatography of the EPS crude polymers synthesized by the strains *Bifidobacterium longum* NB667 and IPLA B667dCo. Carbohydrates in eluted fractions were measured by the phenol-sulfuric acid method. Arrows indicate fractions collected from each elution peak. EPS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

rium.[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

Table 2. Linkage Types (Ratios) Deduced for EPS Produced by the Strains *Bifidobacterium longum* NB667 and *B. longum* IPLA B667dCo[a]

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

[a]Glc, glucose; Gal, galactose; Rha, rhamnose; Hex, hexose; *p*, pyranose ring conformation; *f*, furanose ring conformation.

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 physicochemical 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 noncarbohydrate molecules, the FT-IR spectra in the region $3,750-400$ cm^{-1} was obtained and analyzed (Figure 3). The intensity of bands around 3400, 1400, and 1060 cm^{-1} is due to the hydroxyl stretching vibration of the polysaccharides, and was similar in the peaks of both polymers. The absorption band at 2927 cm^{-1} corresponds to methyl groups and it was also present in this polymer fraction of both microorganisms; since no monosaccharides containing methyl groups were found in this peak, the presence of this FT-IR band may be associated to other noncarbohydrate structures taking part of the polymer. The region between 1860 and 1660 cm^{-1} revealed the presence of carbonyl groups. In this region, some differences were evidenced between the high MM EPS fractions synthesized by the parental and the cholate-resistant strains. Thus, in EPS NB667 a weak absorption band appeared at 1727 cm^{-1} whereas a considerably more intense band appeared at 1685 cm^{-1} in the EPS fraction of the cholate-resistant strain IPLA B667dCo. Some differences were also found at 1209 and 1131 cm^{-1} between fractions of both strains. Moreover, DOSY spectra also corroborated a higher heterogeneity in the peak 1 from EPS IPLA B667dCo than in the peak 1 from EPS NB667 (data not shown). These results indicated that the fractions of high MM found in EPS polymers from the strains *B. longum* NB667 and IPLA B667dCo were equal in monosaccharide content, but presented some differences in the infrared spectra regarding compounds other than carbohydrates that could also be associated to the polymers. The presence of these non-carbohydrate structures may be the reason for the low monosaccharide recovery obtained after the acid hydrolysis of the high MM fraction; this fact and mismatches between the hydrodynamic volume and the true M_w may be contributing to the discrepancies observed between the apparent MM and M_w obtained for peak 1 using different techniques. The elucidation of the chemical nature of these noncarbohydrate residues and the structure of the high MM fraction present in the EPS synthesized by the parental and the cholate derivative *B. longum* strains should take advantage from using nuclear magnetic resonance spectroscopy and mass spectrometry 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 physicochemical 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.*
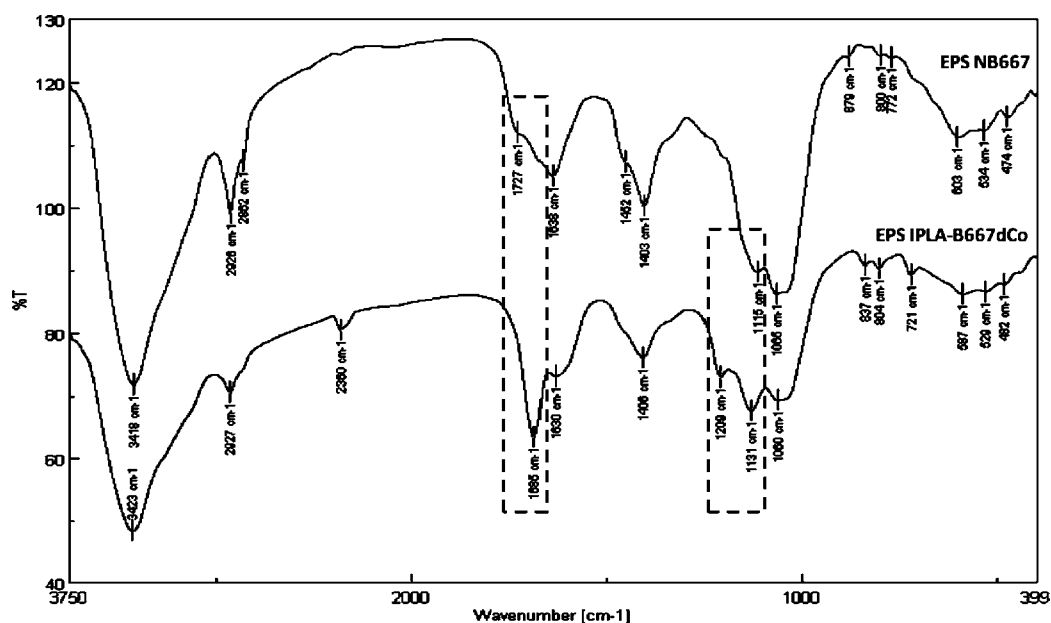
Figure 3. Fourier-transform infrared spectra of the peak of high MM purified by preparative-SEC in Sepharose CL6B from crude EPS polymers of the strains *Bifidobacterium longum* NB667 (upper line) and *B. longum* IPLA B667dCo (bottom line). % *T* = percentage of transmittance. The dotted rectangles indicate areas of the spectrum displaying differences between parental and cholate resistant strains.

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[a]

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
	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]
5	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]

[a]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.

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 physicochemical 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 interindividual 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
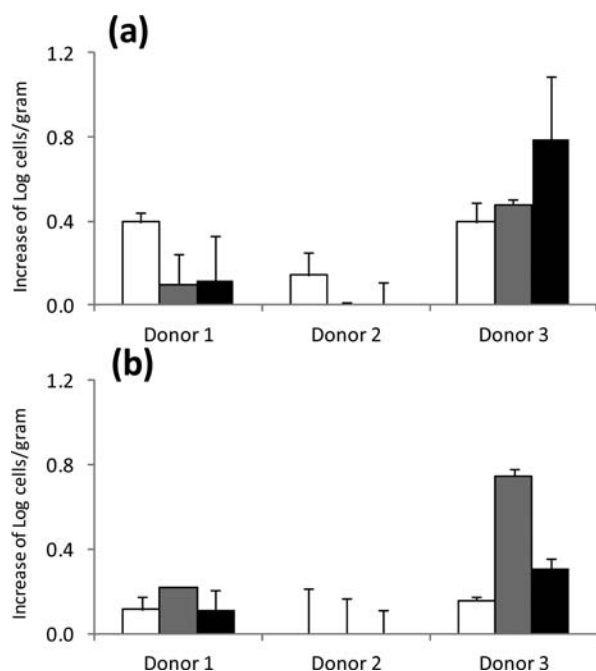

Figure 4. Increase, with respect to time zero, of *Bifidobacterium* counts measured by qPCR in fecal slurry cultures from three human adult donors in the absence of external carbohydrate added (control, white bars) and using crude EPS isolated from the strains *Bifidobacterium longum* NB667 (gray bars) 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.

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 variations in the carbohydrate composition encountered between the polymers of both microorganisms.

Several surface macromolecules of Gram-positive bacteria, among which are glycosylated structures and EPS, could act as signal molecules interacting with eukaryotic cells of the host.[35] Thus, variations in composition, as well as in proportions, between the different monosaccharides and linkage types could modify the structural and functional characteristics of the polymers and hence the possible influence in the cross-talk mechanisms of the producing bacteria with the host. In this

way, we have reported a notable increase in hydrophobicity as well as enhanced in vitro adhesion to human intestinal mucus of the cholate-resistant strain *B. longum* IPLA B667dCo with respect to the parental sensitive strain *B. longum* NB667.[36]

In the current work we have corroborated, by using two strains of the species *B. longum* as a model of study, that the acquisition of bile salt resistance promoted changes in the physicochemical characteristics of EPS. Thus, we suggest that these modifications in EPS production may be a mechanism of response to bile stress although this hypothesis, as well as the ecological relevance of these changes, deserves future confirmation. On the other hand, differences in composition and glycosidic linkages of the polymer fractions produced by parental and cholate-resistant strains were not affecting their capability to be fermented by the intestinal microbiota. To the best of our knowledge, this is the first report on changes promoted by the acquisition of a stable bile-resistance phenotype in the characteristics of EPS produced by micro-organisms of the species *B. longum*.

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