Extraction of the same novel homoglycan mixture from two different strains of *Bifidobacterium animalis* and three strains of *Bifidobacterium breve*.

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Abstract

Three strains of *Bifidobacterium breve* (JCM 7017, JCM 7019 and JCM 2258) and two strains of *Bifidobacterium animalis* subsp. *lactis* (AD011 and A1dOxR) were grown in broth cultures or on plates, and a standard exopolysaccharide extraction method was used in an attempt to recover exocellular polysaccharides. When the extracted materials were analysed by NMR it was clear that mixtures of polysaccharides were being isolated including exopolysaccharides (EPS) cell wall polysaccharides and intracellular polysaccharides. Treatment of the cell biomass from the *B. breve* strains, or the *B. animalis* subsp. *lactis* AD011 strain, with aqueous sodium hydroxide provided a very similar mixture of polysaccharides but without the EPS. The different polysaccharides were partially fractionated by selective precipitation from an aqueous solution upon the addition of increasing percentages of ethanol. The polysaccharides extracted from *B. breve* JCM 7017 grown in HBM media supplemented with glucose (or isotopically labelled D-glucose-1-¹³C) were characterised using 1D and 2D-NMR spectroscopy. Addition of one volume of ethanol generated a medium molecular weight glycogen (Mw = 1 x 10⁵ Da, yield 200 mg/L). The addition of two volumes of ethanol precipitated an intimate mixture of a low molecular weight β -(1→6)-glucan and a low molecular weight β -(1→6)-galactofuranan which could not be separated (combined yield 46 mg/L).

When labelled D-glucose-1-¹³C was used as a carbon supplement, the label was incorporated into >95% of the anomeric carbons of each polysaccharide confirming they were being synthesised *in situ*. Similar ¹H NMR profiles were obtained for polysaccharides recovered from the cells of *B. animalis* subsp. *lactis* AD011 and A1dOxR (in combination with an EPS), *B. breve* JCM 7017, *B. breve* JCM 7019, *B. breve* JCM 2258 and from an EPS (-ve) mutant of *B. breve* 7017 (a non-EPS producer).

Keywords: Probiotics, cellular polysaccharide, exopolysaccharide,

Introduction

Bifidobacterium is an important bacterial genus and, especially in the first few months following birth, it constitutes an important microbiota component of the gastrointestinal tract of healthy humans (Kato et al. 2017, O'Callaghan and van Sinderen 2016). Recently there has been considerable interest in specific strains of bifidobacteria as their presence in the gut microbiota has been linked to a range of health benefits (Arbolea et al. 2016, Orrhage and Nord 2000, Tojo et al. 2014) and a number of strains of bifidobacteria are considered as probiotic organisms (FAO-WHO 2006, Kleerebezem and Vaughan 2009, Picard et al. 2005). The most frequently reported health benefits attributed to bifidobacteria include: protection against pathogenic organisms; immuno-modulation (Fanning et al. 2012, Hidalgo-Cantabrana et al. 2012, López et al. 2012) and improvement of gut barrier function (Hughes et al. 2017), relief of a number of GI-tract disorders (Duranti et al. 2016, Ruas-Madiedo et al. 2006, Saez-Lara et al. 2015) and reduction of cholesterol levels (Bordoni et al. 2013, Choi et al. 2013, Miremedi et al. 2014, Öner et al. 2014). Researchers have investigated the molecular mechanisms underlying the probiotic effect of strains of bifidobacteria and, among others, have focused on their production of exopolysaccharides (EPSs) (Fanning et al. 2012, Hidalgo-Cantabrana et al. 2014). The results of both *in vitro* and *in vivo* experiments suggest specific EPSs are able to interact with intestinal epithelial cells to elicit a biological response. In our previous studies, using EPSs from lactic acid bacteria (Balzaretto et al. 2017, Patten et al. 2014), and in studies undertaken by other research groups with EPSs from bifidobacteria, it has been demonstrated that certain

EPSs can stimulate production of pro- and anti-inflammatory cytokines(Fanning et al. 2012, Hidalgo-Cantabrana et al. 2012, Jones et al. 2014, López et al. 2012). At the same time, results from *in vivo* and *in vitro* experiments have demonstrated that exocellular polysaccharides, including EPS and cell wall-associated polysaccharides (CWAPs) can protect against pathogenic organisms(Fanning et al. 2012, Ruas-Madiedo et al. 2006) and reactive oxygen species by providing a physical barrier to these potentially harmful materials(Xu et al. 2011a, Xu et al. 2011b).

Despite the potential importance of the bacterial exocellular polysaccharides very little is known about the structures of the polysaccharides produced by bifidobacteria. Structural information for only four EPSs from bifidobacteria have been published: two from *Bifidobacterium longum* (Altmann et al. 2016, Inturri et al. 2017, Kohno et al. 2009) and two from *Bifidobacterium animalis* (Leivers et al. 2011, Shang et al. 2013). A further nine papers have described a variety of cell-wall polysaccharides recovered from strains of bifidobacteria (Fischer Werner 1987a, Habu et al. 1987, Nagaoka et al. 1988, Nagaoka et al. 1995, Nagaoka et al. 1996, Tone-Shimokawa et al. 1996, Uemura and Matsumoto 2014, Valueva et al. 2013, Zdorovenko et al. 2009) and the structures of two teichoic acids have also been reported (Veerkamp et al. 1983). The reason why so few bifidobacterial exocellular polysaccharides have been characterised is because these bacteria are often difficult to grow, they require a complex growth medium and they often produce very small amounts of mixtures of polysaccharides. Traditional growth media, MRSc and BIM, include small amounts of polysaccharides that can be isolated along with EPS/CWAP material and these can seriously interfere with the analysis. To overcome this problem, a new growth medium (HBM) which is free from polysaccharide-equivalent material and which supports the growth of bifidobacteria has recently been developed(Alhudhud et al. 2014). Despite the availability of HBM, the recovery of polysaccharides is still problematic and the procedures used to isolate them are lengthy and often provide complex mixtures.

In our laboratories we are working to develop new methods for growing bifidobacteria and for isolating their exocellular polysaccharides. In this paper we describe the results of a series of experiments where we have grown strains of *B. breve* and *B. animalis* in HBM media and investigated a number of the steps involved in the isolation procedure in order to determine conditions for recovering clean samples of exocellular polysaccharides. The focus of the present paper is on the characterisation of the polysaccharides that are extracted directly from the cell biomass from strains of bifidobacteria that have been treated with aqueous alkali; strains studied include *B. breve* JCM 7017, *B. breve* JCM 7019 *B. breve* JCM 2258, *B. animalis* subsp. *lactis* AD011, *B. animalis* subsp. *lactis* A1dOxR, and also an EPS (-ve) mutant of *B. breve* 7017. Characterisation of the EPSs is ongoing and will be reported separately.

Materials and methods

Bacterial strains and starter culture preparation

The strain *B. animalis* subsp. *lactis* AD011 was isolated from a commercial dairy product, the identification of the strain and species has been described previously(Alhudhud et al. 2014). A sample of the polysaccharide recovered from *B. animalis* subsp. *lactis* A1dOxR was a kind gift from IPLA-CSIC (Asturias, Spain). *B. breve* sp. JCM 7017, JCM 7019 and JCM 2258 were purchased from the Japan Collection of Microorganisms (RIKEN BioResource Center, Ibaraki, Japan). The mutant strain of the *B. breve* 7017 EPS (-ve), is a direct derivative of *B. breve* JCM 7017 in which the gene encoding the predicted priming glycosyl transferase was disrupted by

site-directed mutagenesis (the gene with locus tag B7017_0382 and mutagenesis methodology are described in Bottacini et al., (Bottacini et al. 2014)). The details of the EPS-negative mutant construction and phenotypic characterisation is to be described elsewhere. A sample of *Lb. paracasei* subsp. *paracasei* DG was a kind gift from the Dr S Guglielmetti at the Università degli Studi di Milano (Italy), a sample of *Lb. salivarius* NCIMB 8818 was purchased from the National Collection of Industrial, Food and Marine Bacteria (NCIMB, Aberdeen, UK). Once received, cultures were stored on beads (Microbank, Pro-Lab diagnostics Ltd, Neston UK) at -80 °C.

Bacterial growth and extraction protocols employed with batch cultures.

Prior to fermentations, cultures were revived on MRS (with cysteine) plates (LabM Ltd, Heywood, UK) incubated anaerobically (80% N₂, 10% CO₂ and 10% H₂), in an anaerobic cabinet (Don Whitley Anaerobic Workstation, Shipley, UK) at 37 °C for 48 h. Cultures were passaged twice prior to sub-culturing into 500 mL of HBM(Alhudhud et al. 2014) containing either glucose or 1-¹³C-labelled D-glucose . The fermentation was typically allowed to continue for 48 to 60 h. To separate the exocellular polysaccharide produced during bacterial growth, at the end of the fermentation cultures were centrifuged (10,000 x g, 15 min, 4 °C) (Avanti J- 26S XPI-Beckman Coulter, Inc., Indianapolis, USA) to leave bacterial cells as a pellet and a solution of exocellular polysaccharide as the supernatant. To recover the CWAPs, several procedures were used (see below) the final optimised procedure was as follows: the cell pellet was resuspended in NaOH (1.0 M) and was stirred overnight at room temperature before it was centrifuged (10,000 x g, 15 min, 4 °C) to remove bacterial cells. Polysaccharides were fractionally precipitated from the aqueous solution by the addition of ethanol. After the addition of one volume of ethanol the resulting suspension was allowed to stand for 24 h before collecting a first precipitate (fraction-**C1**) which was collected by centrifugation (10,000 x g, 15 min, 4 °C). A second volume of ethanol was then added to the supernatant which yielded a second precipitate, fraction-**C2**, which was collected in the same way. The precipitated fractions were resuspended in ultra-pure water, dialysed in 12-14 kDa MWCO cellulose membranes (Sigma, Dorset, UK) against water for 3 days at 4°C and finally freeze-dried to obtain the polysaccharide fraction.

Attempted CWAPs extraction protocol using NaCl

Cells were washed with PBS (pH 7.0) and then suspended in a NaCl solution (1M) and sonicated (40 W, 3 min at 4 °C) to release polysaccharides from the cell wall. The resulting suspension was centrifuged (10,000 x g, 10 min at 4 °C) to remove cells and any insoluble material. The CWAPs were precipitated from the supernatant with addition of two volumes of chilled ethanol and the sample was left for 48 h at 4 °C to allow for complete precipitation. After 48 h CWAPs were collected by centrifugation (25,000 x g, 30 min at 4 °C) then dialysed for three days against deionised water with three changes of water per day. Finally, the dialysed fractions were freeze-dried.

Attempted CWAPs extraction protocol using EDTA

The cells collected by centrifugation were suspended in an aqueous solution of EDTA (0.05 M) and were stirred gently for 4 h at 4 °C. The CWAPs were recovered from the suspension using exactly the same procedures as described above in the NaCl method.

Use of SEC-MALLS to determine the purity of the polysaccharides extracted from fermentations.

The purity of polysaccharides was determined by SEC-MALLS. For SEC-MALLS, solutions of polysaccharides (1 mg mL^{-1}) were prepared in aqueous sodium nitrate (0.1 M) and left for 24 h to completely dissolve. Samples ($100 \text{ }\mu\text{L}$) were injected onto an analytical SEC system comprising three columns connected in series: PL- Aquagel-OH 40, 50 and 60 ($8 \text{ }\mu\text{m}$ particle size, $30 \text{ cm} \times 7.5 \text{ mm}$, Agilent, Cheadle, UK). The neutral analytes were eluted with aqueous sodium nitrate (0.1 M) flowing at 0.7 mL min^{-1} . The concentration of the polysaccharides eluting from the column were determined using a differential refractometer (Optilab rEX, Wyatt technology, Santa Barbara, CA, USA) and the weight average molecular mass was measured using a Dawn-EOS MALLS operating with a 690 nm laser (Wyatt technology). The concentrations of proteins and nucleic acids were monitored using in-line UV detection (Shimadzu, Milton Keynes, UK).

Monomer composition, determination of absolute configuration and linkages of the C1 and C2 fractions

The monomer composition of the *B. breve* JCM 7017 **C1** and **C2**-fractions was determined after acid hydrolysis. The polysaccharides (1.5 mg mL^{-1}) were treated with 2 M TFA (120°C for 2 h) and the identity of the released monomers was determined using high performance anion exchange chromatography (HPAEC) on a Thermofisher ICS3000 ion chromatography system (Sunnyvale CA, USA) equipped with a CarboPac PA20 column ($150 \text{ mm} \times 3 \text{ mm}$). Monomers were eluted using a 10 mM sodium hydroxide mobile phase at a flow rate of 0.3 mL min^{-1} and detected using a pulsed amperometric detector (PAD) operating with a dual potential waveform.

The absolute configuration of the monomers was determined using Gerwig's method (Gerwig et al. 1978). In summary, the polysaccharides (0.2 mg) were heated at 120°C with an aqueous solution of TFA ($0.5 \text{ mL } 2 \text{ M}$) for 30 min. The sample was evaporated to dryness under a stream of nitrogen. The solid residue was dissolved in (+)-2-butanol (0.2 mL) and a small portion of acetyl chloride ($15 \text{ }\mu\text{L}$) was added and the vessel was purged with dry nitrogen for 30 sec and was then heated at 80°C for 16 h. After the reaction was complete the solution was evaporated to dryness under a stream of nitrogen, methanol ($0.5 \text{ mL} \times 2$) was added and the sample evaporated to dryness. Finally, the sample was acetylated with a mixture of acetic anhydride (0.2 mL) and pyridine (0.1 mL), whilst heating at 120°C for 30 min. The acetylated products were partitioned between H_2O (0.5 mL) and EtOAc (0.5 mL) and the ethyl acetate layer was analysed by GC-MS. The samples were eluted from a HP-5 column ($30 \text{ m} \times 0.25 \text{ mm-id}$, $0.25 \text{ }\mu\text{m}$ film) using helium as carrier (9 psi, flow rate 1 mL min^{-1}) and using the following temperature programme: start temperature 180°C , hold time 1 min, and a final column temperature of 220°C reached via a rising gradient of 1°C min^{-1} .

For linkage analysis, the *B. breve* JCM 7017 **C1** and **C2** fractions were permethylated using the procedures described by Stellner et al. (Stellner et al. 1973). The methylated-polysaccharide was hydrolysed by treatment with 2 M TFA (120°C for 2 h) and the methylated monosaccharides converted to their corresponding methylated alditol acetates using NaBD₄ as the reducing agent. The identity of the variously methylated alditol acetates was determined by GC-MS and by analysis of the individual fragmentation patterns observed in the MS. GC-MS analyses were performed on an Agilent 7890A GC system (Santa Clara, CA, USA) coupled to an Agilent 5975B MSD quadrupole MS. The samples were eluted from a HP-5 column ($30 \text{ m} \times 0.25 \text{ mm-id}$, $0.25 \text{ }\mu\text{m}$ film) using helium as carrier (9 psi, flow rate 1 mL min^{-1}) and using the following temperature programme: start temperature 140°C , hold time 1 min, and a final column temperature of 220°C reached via a rising gradient of $2.5^\circ\text{C min}^{-1}$.

NMR analysis of polysaccharides

For NMR analysis polysaccharide samples were dissolved (10 mg mL⁻¹) directly in D₂O (Goss Scientific Instruments Ltd., Essex, UK). NMR spectra were recorded at a probe temperature of 70°C. All of the NMR spectra were recorded on a Bruker Avance 500.13 MHz ¹H (125.75 MHz ¹³C) spectrometer (Bruker-biospin, Coventry, UK) operating with Z-field gradients where appropriate, and using Bruker's pulse programs. Chemical shifts are expressed in ppm relative to either internal or external acetone; δ 2.225 for ¹H and δ 31.55 for ¹³C. The 2D gs-DQF-COSY spectrum was recorded in magnitude mode at 70°C. The TOCSY experiment was recorded with a mixing time of 120 ms. The 2D-heteronuclear ¹H-¹³C HSQC spectrum was recorded using Bruker pulse sequences and 256 experiments of 1024 data points. For the majority of spectra, time-domain data were multiplied by phase-shifted (squared-) sine-bell functions. After applying zero-filling and Fourier transformation, data sets of 1024-1024 points were obtained.

Results

Isolation of polysaccharides.

In order to recover as much polysaccharide as possible, the extraction procedure was reduced to a minimum number of steps and deliberately excluded steps that would normally be used to remove proteins. In provisional experiments, the cell pellet recovered from either plates or from broth cultures was treated with a range of different reagents in an attempt to extract CWAPs including EDTA, sodium chloride and sodium hydroxide. The highest yield (approximately 300 mgL⁻¹) of material was recovered when cells were left stirring overnight in an aqueous alkaline solution (1M). After centrifugation, polysaccharides were precipitated from the supernatant on the addition of ethanol. NMR spectra recorded for the precipitated materials (data not shown) suggested that complex mixtures of polysaccharides were being recovered. However, it was clear from inspection of the NMR spectra that a number of resonances in the anomeric region were the same in material collected from the *B. breve* (JCM 7017, JCM 7019, JCM 2258) and *B. animalis* subsp. *lactis* (A1dOxR and AD011) strains suggesting that the same polysaccharides were being produced by the different strains, including the EPS (-ve) mutant. In subsequent experiments it was found that it was possible to partially fractionate the different polysaccharides by gradually increasing the percentage of ethanol that was being added. Addition of one volume of chilled ethanol produced, after exhaustive dialysis, a glycogen-rich fraction labelled **C1** (typically 100 mg from a 500 mL fermentation). Addition of a second volume of ethanol released a small amount of polysaccharide (20-25 mg from a 500 mL fermentation), labelled as fraction **C2**, which was subsequently identified as a mixture of a β-glucan and a β-galactafuranan. When the same experiments were performed with two control strains (*Lb. paracasei* DG and *Lb. salivarius* NCIMB 8818) very small amounts of material were recovered in both the **C1** and **C2** fractions.

Characterisation of the polysaccharides present in the C1-fraction from *B. breve* JCM 7017.

The methods that were used to characterise the molecular structure of the polysaccharide present in the various **C1**-fractions included NMR spectroscopy, monomer analysis, linkage analysis and determination of the absolute configuration of the sugars. Monomer analysis identified glucose as the only monosaccharide present in **C1** (>95%). Absolute configuration analysis using Gerwig's method (Gerwig et al. 1978)

confirmed that the polysaccharide was composed of D-glucose. Finally, linkage analysis identified a 1,4-linked hexose and a small amount of a 1,4,6-linked hexose. The monomer, linkage and absolute configuration results are consistent with the **C1**-fraction being a 1,4-linked-D-glucan with a small degree of branching. When monomer analysis was performed with the material recovered from the fermentation employing D-glucose-1-¹³C as a carbon feed, C-1-deuterioalditol acetates were isolated and the isotope patterns observed in the GC-MS for the 1-deuterio-C1-¹³C-alditol acetates (comparison of fragments including ¹²CH₂OAc from position-6 with natural abundance isotope at M+1 & with M+2 fragments including ¹³CHDOAc at position-1) confirmed that greater than 95% of the glucan was being generated *in situ*.

The proton NMR spectrum of the **C1**-fraction was recorded and compared to that of a solution of a starch standard (Fig 1a). A series of 2D-NMRs were also recorded (not shown) and these confirmed that the main polysaccharide present in the **C1**-fraction was a bacterial glycogen.

Figure 1a. 500-MHz ¹H-NMR recorded in D₂O at 70 °C, (top spectrum) selected region of the spectrum of a starch standard; (middle-spectrum) selected region of the polysaccharide material recovered after treatment of the biomass generated during the growth of *B. animalis subsp. lactis* AD011 with sodium hydroxide (1 M) and precipitated with one volume of ethanol (**C1**-Fraction) (bottom spectrum) polysaccharide material recovered after treatment of the biomass generated during the growth of *B. breve* JCM 7017 with sodium hydroxide (1 M) and precipitated with one volume of ethanol (**C1**-Fraction).

289 A number of other peaks were visible in the NMR spectrum and the majority of these
 290 correlate with those observed for the **C2**-fraction suggesting that the fractionation
 291 process has not completely resolved the different polysaccharides. The purity and the
 292 molecular mass of the samples was examined using SEC-MALLS coupled to both a
 293 refractive index (RI) and UV-detectors. A very broad peak corresponding to a medium
 294 molecular weight material eluted between 25-35 min (Fig. 2a), a similar broad peak was
 295 observed in the chromatograph generated by the refractive index detector and the
 296 asymmetric profile suggests that two different populations of material were eluting (Fig
 297 2b) and the weight average mass of the combined glycogen peak was measured as 1.0×10^5 g/mol. These could potentially relate to different polymers with different degrees of
 299 branching within a bacterial glycogen. The refractive index trace also contains a late
 300 eluting peak which has a significant UV chromophore (UV trace not shown) and this is
 301 likely to be from a small amount of protein that is present in the sample.

302
 303 **Figure 2.** SEC-MALLS chromatographs obtained for polysaccharides extracted after treatment
 304 of the cells of *B. breve* JCM7107 with sodium hydroxide (1M) (a) light scattering detector
 305 response during elution of the **C1**-fraction (b) Refractive index detector response during elution
 306 of the **C1**-fraction (c) UV (280 nm) detector response during elution of the **C2**-fraction (d)
 307 refractive index detector response during elution of the **C2**-fraction.

308 Characterisation of the polysaccharides present in the **C2**-fraction.

309 In all bacterial strains studied, the amount of material recovered in the **C2**-fractions was
 310 smaller than that in the **C1**-fractions (typically 20-25 mg from a 500 mL fermentation).
 311 The composition of the material was examined using SEC-MALLS which included RI
 312 and UV detectors. The chromatograph generated by the light scattering detector did not
 313 show any significant peaks implying that only relatively low molecular weight materials
 314 (50-5 kDa) were present in the **C2**-fraction. However, peaks were visible in both the RI
 315 and UV traces which suggested that a mixture of low molecular weight polysaccharides
 316 and proteins was being recovered (Fig 2c & 2d). In the RI trace three distinct and
 317 closely eluting peaks were visible (Fig 2c); the final peak has a significant UV
 318 chromophore (Fig 2d) and this is likely to be protein. The other two peaks do not absorb

319 UV and do not scatter light to any appreciable extent, and are therefore likely to be low
320 molecular weight polysaccharides.

321
322 **Figure 1b.** Selected region of the 500-MHz ^1H -NMR spectrum recorded in D_2O at 70°C of the
323 polysaccharide recovered from the biomass generated during the growth of *B. breve* JCM 7017
324 after treatment with sodium hydroxide (1 M) and precipitation with two volumes of ethanol (**C2**-
325 Fraction).

326 In an attempt to determine the structures of the low molecular weight polysaccharides,
327 ^1H -NMR spectra were recorded for a number of different **C2**-fractions. From inspection
328 of the ^1H -NMR spectrum (Fig 1b) it was clear that two principal sets of anomeric signals
329 were present: the first set at 5.1-5.0 ppm & the second at 4.55-4.35 ppm. The same ^1H -
330 NMR anomeric profile was recorded for the **C2**-fractions recovered from the four *B.*
331 *breve* strains (including the *B. breve* 7107 EPS (-ve) mutant) and the two *B. animalis*
332 strains. However, the relative integral areas for the two sets of protons differed for the
333 different strains and also for the **C2**-fractions recovered from different fermentations.
334 These results, when taken in combination with the SEC-MALLS data, suggest that two
335 different low molecular weight polysaccharides were being produced. Unfortunately, all
336 attempts to separate the two using preparative size exclusion chromatography and
337 fractional precipitation failed. Monosaccharide analysis identified that the **C2**-fractions
338 contained galactose and glucose, and that the ratio between galactose and glucose
339 varied between 1:1.05 and 1:2.0 for the different C2-fractions; trace levels of rhamnose
340 (<2%) were observed in some samples. Linkage analysis was performed on two **C2**
341 batches, both of which contained a small amount of the **C1** material. The GC-MS trace
342 recorded during the linkage analysis indicated the presence of six unique methylated
343 alditol-acetates including: (1) a small peak for 1,5-di-O-acetyl-2,3,4,6-tetra-O-
344 methylhexitol, which is consistent with a terminal hexopyranose; (2) a very small peak
345 for 1,4-di-O-acetyl-2,3,5,6-tetra-O-methylhexitol, which is consistent with a terminal
346 hexofuranose; (3) a small peak for 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylhexitol, which is
347 consistent with either a 1,4-linked-hexopyranose or a 1,5-linked-hexofuranose; (4) a
348 second 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylhexitol, which is also consistent with either a
349 1,4-linked-hexopyranose or a 1,5-linked-hexofuranose; (5) a large peak for 1,5,6-tri-O-
350 acetyl-2,3,4-tri-O-methylhexitol, which is consistent with a 1,6-linked-hexopyranose; (6)

a peak for a 1,4,6-tri-O-acetyl-2,3,5-tri-O-methylhexitol, which is consistent with a 1,6-linked-hexofuranose. No branching points were detected in the linkage analysis which suggests that only linear polysaccharide chains are present. Absolute configuration analysis confirmed that the polysaccharides were composed of D-galactose and D-glucose.

Use of 1D- and 2D-NMR to determine the structures of the polysaccharides present in the C2-fraction from *B. breve* JCM 7017.

In order to gain more information about the nature of the material being recovered in the C2-fraction, a series of 2D-NMR spectra were recorded for the C2-fractions, including a ^1H - ^{13}C HSQC spectrum (Fig 3). The interpretation started by looking at the largest anomeric resonances including the anomeric signal at 5.06 ppm which was attached to a carbon resonating at a high chemical shift (108 ppm) which is characteristic of a β -hexofuranose residue and this was subsequently identified as belonging to a β -galactofuranan (see below). The second set of large anomeric proton resonances appear as a series of overlapping doublets centred at 4.5 ppm (Fig 3. F2-axis, 4.55-4.45 ppm); these were subsequently identified as belonging to a β -glucan (see below).

Figure 3. ^1H - ^{13}C -HSQC spectrum recorded in D_2O at 70 °C at 500 MHz for the *B. breve* JCM 7017 C2-Fraction. Labels identifying ring hydrogen/carbon positions (H/C-1 to H/C-6).

Using the combination of the COSY (Fig 4a) and TOCSY spectra (Fig 4b) it was possible to follow the scalar coupling of the dominant anomeric resonances from H-1 through to H-6 and to identify the location of the ring proton resonances: these are listed in Table 1 and the identity of the hydrogens for the most prominent resonances are highlighted on the HSQC spectrum (Fig 3). The location of the corresponding carbons, C-1 to C-6, was identified from inspection of a combination of a HSQC spectrum (Fig 3) and a HSQC-TOCSY spectrum (not shown). For the hexofuranose, starting with C-1 at 108.4 ppm, the chemical shifts for each of the carbons and hydrogens are very close to those that have previously been published for a β -D-(1 $\rightarrow$ 6)-galactofuranan (Bertin et al. 2013). For the second set of resonances, the

381 chemical shifts for each of the carbons are very close to those that have previously
 382 been published for a β -D-(1 $\rightarrow$ 6)-glucan(Saitô et al. 1977).

383

384 **Table 1.** ^1H and ^{13}C chemical shifts in ppm recorded for the **C2**-fraction of *B. breve* JCM 7017
 385 at 70 °C in D₂O. ND-identifies resonances which could not be detected: mainly due to overlap
 386 with larger peaks.

387

Chemical shifts of monosaccharide resonances (ppm)						
Residue	C-1 H-1	C-2 H-2	C-3 H-3	C-4 H-4	C-5 H-5	C-6 H-6
-6- β -D-Galf-1	108.4 5.04	81.5 4.11	77.4 4.06	83.8 4.00	70.1 3.90	69.7 3.85 & 3.62
Reference -6- β -D-Galf-1	109.0 5.05	82.1 4.12	78.0 4.07	84.5 4.01	70.8 3.97	70.2 3.86 & 3.65
-6- β -D-Glcp-1	103.4 4.49	73.5 3.32	76.2 3.48	70.3 3.44	75.4 3.61	69.4 4.19 & 3.86
Reference -6- β -D-Glcp-1	104.2	74.2	76.8	70.6	76.1	70.0
R- β -D-Galp-1-Gro	103.6 4.40	71.7 3.54	69.7 3.63	70.4 3.94	ND ND	ND ND

388

389

390 **Figure 4 (a)** ^1H - ^1H -COSY spectrum recorded at 500 MHz in D₂O and at 70 °C for the *B. breve*
 391 JCM 7107 **C 2**-fraction

Figure 4 (b) ^1H - ^1H -TO CSY spectrum recorded at 500 MHz in D_2O and at 70 °C for the *B. breve* JCM 7107 **C2**-fraction (R- β -D-Galp= The reducing end terms of the galactofuranan).

4. Discussion

The extraction procedures commonly employed (Ruas-Madiedo and de los Reyes-Gavilan 2005) to recover EPS and CWAPs are lengthy and often result in the recovery of small amounts of complex mixtures of polysaccharides. Our previous studies have shown that this is particularly true when working with bifidobacteria (Leivers et al. 2011) and a similar situation was observed in the experiments reported here; the yields of polysaccharides recovered were very small and both the NMR spectra and the SEC-MALLS traces of the crude products showed that mixtures of polysaccharides were being isolated. When such small amounts of material are being isolated it is extremely important to identify if the polysaccharides are being produced *in situ* and that they are not being added as media components. In the current work this was achieved by using a ^{13}C -labelled carbon feed and the incorporation of the ^{13}C -label into each of the polysaccharides was measured confirming their *in situ* biosynthesis.

The fractionation of bacterial polysaccharides by selective precipitation of components with ethanol has previously been reported (Dueñas-Chasco et al. 1998) and the application of the technique, in the studies described above, gave two distinct fractions **C1** and **C2** which were amenable to analysis. The various NMR spectra recorded for the **C1**-fraction confirmed that the material was a bacterial glycogen with a medium molecular weight. Whilst the scientific literature contains a significant number of references to bacteria that can produce glycogen (Shively 1974), as far as we are aware, this is the first report of the isolation of a soluble glycogen from a bifidobacteria. Previous reports of bacterial glycogen, including an insoluble glycogen-like material isolated from *B. bifidum* ssp. *pennsylvanicum* (Veerkamp et al. 1983), have identified it as being an intracellular storage polysaccharide and therefore the presence of glycogen

in the **C1**-fraction is likely to be a result of a significant amount of cell lysis occurring either in the fermentation or during the extraction process.

Analysis of the NMR of the **C2**-fraction identified the presence of two further homoglycans: a β -D-(1 $\rightarrow$ 6)-galactofuranan and a β -D-(1 $\rightarrow$ 6)-glucan. One feature of the NMR spectra recorded for all **C2**-fractions that remains to be explained is why a series of overlapping doublets are observed for the anomeric proton of the β -glucan. Variable decoration of the backbone would account for the multiple doublets, but this possibility can be ruled out by the linkage analysis which excludes any branching and by our failure to observe substituents, esters or ethers etc, in the NMR. In the linkage analysis, peaks (1, 2, 5 and 6) are consistent with the presence a β -D-(1 $\rightarrow$ 6)-galactofuranan and a β -D-(1 $\rightarrow$ 6)-glucan. The ratio of the peak areas for the terminal hexopyranose (peak 1) with that for the 1,6-linked hexopyranose (peak 5) suggests that the β -D-(1 $\rightarrow$ 6)-glucan has a low molecular weight (this assumes that the small amount of residual glycogen (responsible for peak 4) has a high molecular weight similar to that observed in the **C1**-fraction). In contrast, the ratio of the peak areas for the terminal hexofuranose (peak 2) with that for the 1,6-linked hexofuranose (peak 6) suggests that the (1 $\rightarrow$ 6)-galactofuranan has a higher molecular weight. If the β -D-(1 $\rightarrow$ 6)-glucan does have a relatively low DP then this might explain why overlapping doublets are observed for the anomeric proton resonances: the proximity to the reducing and non-reducing termini determining the absolute chemical shifts of the anomeric protons. Finally, it should be noted that the third peak observed in the monomer analysis (peak 3) did not correlate with the structures deduced from the NMR spectrum.

A second question which needs addressing is why neutral low molecular weight polysaccharides should be found associated with the cells and are not released as EPSs. The fact that the *B. breve* 7017 EPS (-ve) mutant which lacks the gene coding for the priming glycosyl transferase which is needed for EPS synthesis is capable of producing the same material suggests that the polysaccharides are not EPSs that are tightly associated with the cell surface and are being retained with the cells after centrifugation. An indication about where the polysaccharides may come from can be found in work published by Op den Camp et al (Op Den Camp et al. 1984) who reported that *B. bifidum* produces an intimate mixture of two lipoteichoic acids: one with a β -D-(1 $\rightarrow$ 5)-galactofuranan backbone (**1a**) and the second with a β -D-(1 $\rightarrow$ 6)-glucan backbone (**1b**).

However, in later work, Fischer *et al* (Fischer W. 1987b, Fischer W. et al. 1987) suggested that *B. bifidum* actually produces a single lipoteichoic-acid-like macromolecule (**2a**) which in solution with aqueous alkali undergoes hydrolysis to generate the linear two block polysaccharide (**2b**).

H-[5)-β-D-Galp-(1→]_n[-6)-β-D-Glcp-(1→]_m-6)-β-D-Galp(1→3)-Gro (**2b**)

The highly alkaline pH used in the extraction experiments reported here would be expected to rapidly hydrolyse phosphate linkages and to remove lipid fatty acids. It is possible that the low molecular weight polysaccharides isolated in the **C2**-fraction may originally have been present as fragments of either a lipoteichoic-acid-like molecule or as two lipoteichoic acids which have been released during the extraction procedure. If this was the case, then the NMR spectra would be expected to include signals derived from either glycerol or a 1,6-linked-galactopyranosylglycerol located at the reducing terminus. On closer inspection of the proton spectrum there is an additional low intensity anomeric resonance at 4.40 ppm which is potentially a reducing end 6-linked hexose in the β-configuration (Bock and Pedersen 1974). Using a combination of the COSY and TOCSY spectra it was possible to track the scalar coupling but only from H-1 through to H-4 (typical of a galactose residue); the corresponding carbons were obtained from the HSQC spectrum. The position of these C-1 & H-1 resonances perfectly match those published for 1-O-β-galactopyranosylglycerol (Wei et al. 2013). Unfortunately, because of signal crowding in the 3.5-3.9 ppm region it wasn't possible to locate any low intensity glycerol proton resonances. Despite many attempts, the low amount of available material and the quality of the spectra means that it wasn't possible to establish by NMR any link between the glucan and galactofuranan fragments. The most convincing evidence for the presence of two separate low molecular weight polysaccharides is the observation of separate peaks in the SEC-MALLS chromatographs.

5. Conclusion

The results reported here demonstrate that when applying a common EPS extraction procedure to a number of different strains of bifidobacteria multiple polysaccharides are recovered. A mixture of bacterial glycogen, a β-D-(1→6)-galactofuranan and a β-D-(1→6)-glucan was recovered directly from the cells of several *B. breve* and *B. animalis* strains after exposure to aqueous alkali. The strong alkali used in the extraction process is likely to be responsible for releasing the polysaccharides. The β-glucan and β-galactofuranan are likely to be derived from the hydrolysis of phosphate linkages in cell wall associated lipoteichoic acids. It is possible that the bacterial glycogen is an intracellular polysaccharide released by cell lysis during the extraction procedure. The isolation of a mixture of three homoglycans in the present work highlights the importance of identifying the different components present in glycan mixtures in advance of reporting their biological activity. Further work is ongoing to study the biological activity of these different **C2**-fractions.

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