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Title	Structural investigation of polysaccharides extracted from four Bifidobacterium breve strains
Authors	Vinogradov, Evgeny;Sadovskaya, Irina;McDonnell, Brian;Coughlan, Keith;Bottacini, Francesca;van Sinderen, Douwe
Publication date	2026-05-05
Original Citation	Vinogradov, E, Sadovskaya, I, McDonnell, B, Coughlan, K, Bottacini, F & van Sinderen, D 2026, 'Structural investigation of polysaccharides extracted from four Bifidobacterium breve strains', Carbohydrate Research, vol. 566, 109946, pp. 1-10. https://doi.org/10.1016/j.carres.2026.109946
Type of publication	Article (peer-reviewed)
Link to publisher's version	https://www.scopus.com/pages/publications/105037432703 - 10.1016/j.carres.2026.109946
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Item downloaded from	https://hdl.handle.net/10468/18822

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Carbohydrate Research 566 (2026) 109946

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Structural investigation of polysaccharides extracted from four *Bifidobacterium breve* strains

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ARTICLE INFO

Keywords:
Probiotic
Gut microbiota
Polysaccharide
2D NMR
Microbe-host interaction

ABSTRACT

Bifidobacterium breve is a common component of the human gut microbiota, particularly in infancy. Its surface-exposed polysaccharides may be important for its purported health-promoting activities and may serve various functions such as immune evasion and modulation, environmental stress protection, or carbohydrate substrate provision for other gut commensals. In the present work, we extracted and analysed polysaccharides from four strains of *B. breve*. Their chemical structure was established by 2D NMR spectroscopy and methylation analysis. Most analysed strains produced several distinct saccharidic structures, some of which contained common rhamnan substructures. The uncovered complexity and multitude of polysaccharides produced by representatives of a single species of the *Bifidobacterium* genus illustrates clear strain specificity which may underpin probiotic and other properties of this group of human gut commensals.

1. Introduction

Species of the genus *Bifidobacterium* are widely reported to be amongst the first colonisers of the infant human gut [1] and, as such, represent an abundant and keystone component of the early life human gut microbiota. Particular members of this genus, being identified as Gram-positive, non-motile, non-spore forming bacteria with a typical bifurcated morphology, have been reported to play a wide range of beneficial roles in the human digestive tract. These include (but are not limited to) metabolism of indigestible carbohydrates [2], immune function modulation (reviewed by 3), competitive exclusion of pathogens [4,5], vitamin production [6], and production of short-chain fatty acids [7]. They are correlated with positive health outcomes in infants reviewed by Ref. [8] and are consequently commercially exploited as probiotics aimed at reducing the risk or alleviating the symptoms of a number of immunological or gastrointestinal conditions in children and adults (reviewed by 9). Various bifidobacterial species are associated with the human gastrointestinal tract, including *Bifidobacterium longum* (various subspecies), *Bifidobacterium bifidum*, *Bifidobacterium pseudocatenulatum* and *Bifidobacterium breve* [10]. Of these, *B. breve* has arguably been subject to most detailed genetic/genomic studies due to the depth and breadth of work performed using the *B. breve* prototype strain UCC2003 [11–16].

Extracellular polysaccharides produced by Gram-positive bacteria are generally categorised as either cell wall-associated polysaccharides (CWPS; covalently bound to the wall peptidoglycan), exopolysaccharides (EPS; loosely bound to or secreted by the cell), wall teichoic acids (WTA; covalently attached to the peptidoglycan), lipoteichoic acids (LTA; bound by a glycolipid anchor to the phospholipid membrane), or indeed the peptidoglycan itself (reviewed by 17). Of these rather broad categories of macromolecules, only EPS has been categorically shown to be produced by bifidobacteria, and the genetic components of its biosynthesis been conclusively identified [18–20]. The EPS produced by *B. breve* UCC2003 has been shown to be required for long-term colonization in a murine host, by preventing an Ig-mediated adaptive immune response, and to exclude a particular

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https://doi.org/10.1016/j.carres.2026.109946
Received 27 February 2026; Received in revised form 27 April 2026; Accepted 27 April 2026
Available online 28 April 2026
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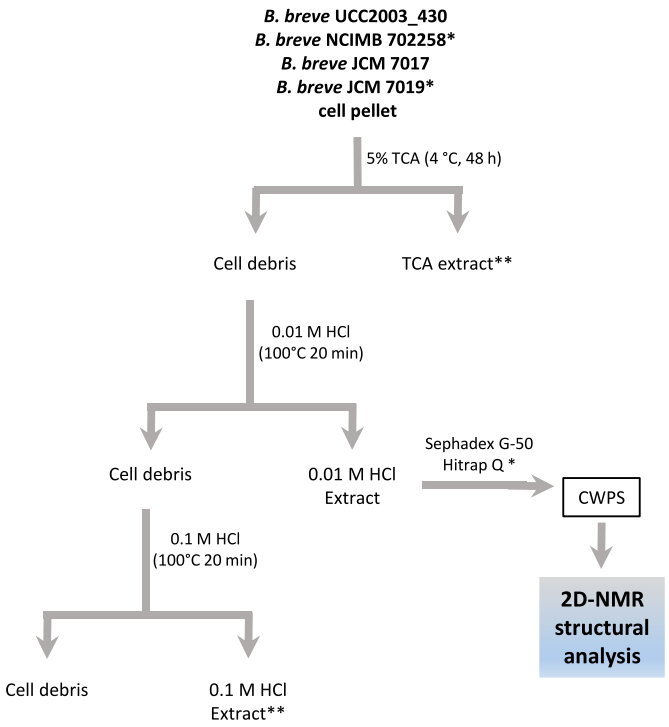


Fig. 1. Schematic representation of extraction and purification procedures employed for the preparation of CWPS samples from different *B. breve* strains. For strains marked with * the CWPS preparations were re-purified by anion-exchange chromatography on a Hitrap Q column. **, extracts were contaminated with presumed intracellular glycogen.

bacterial pathogen [18]. In addition, the EPS of the same strain has been shown to mediate an amelioration of detrimental intestinal cell 'shedding', a process associated with inflammatory bowel disease (IBD; [21]). More recently, the EPS molecule itself has been shown to be metabolised by other gut microbiota in an infant colonic model [22]. Despite these advancements, molecular characterisation of extracellular polysaccharides produced by this species remains understudied, largely due to the advanced techniques (and limitations thereof) required to characterise these important, yet structurally complex [19,20] saccharidic macromolecules. Hypothetical non-EPS bifidobacterial extracellular polysaccharides have in general been less well characterised, not only due to a lack of genetic studies, but also due to the difficulties in identifying the precise structure of the molecule(s) which can easily be conflated with substructures of other cell wall-associated molecules [23].

A number of structural studies have shed light on predicted EPS and possible CWPS produced by bifidobacteria, which a recent comprehensive review [24] has collated effectively. Of note, despite the genus' prominent role in the gut, extracted polysaccharides (here referred to as PSs) structures produced by only thirteen bifidobacterial strains have been determined, highlighting the need for further exploration of these molecules and the corresponding genetics and biochemistry of their synthesis. As mentioned above, however, the determination of these structures is somewhat hampered by the technical expertise required, inconsistencies in approaches (to date), and the harsh treatments of cell walls needed to isolate the PSs themselves. This harsh treatment, usually requiring the use of strong acids, frequently leads to fragmentation of the PS structures [25]. This can lead to difficulty in identifying the original or native structure of the CWPS/EPS under investigation, as distinct from other structures such as teichoic acid components and carbohydrate decorations, which may be cleaved from the main glycan backbone [26–28]. Adding to this complexity may be the presence of glycogen, an intracellular storage carbohydrate [29] which can be released from the cell cytoplasm upon lysis [23,27].

To advance the complex field of bifidobacterial cell surface polysaccharides, it is necessary to attempt to selectively isolate and determine the structure of distinct extracellular polysaccharide molecules from the cell surfaces of these important bacteria, which may then be further explored by genetic interrogation. In the present work we isolated and characterized extracellular polysaccharides from four strains of *B. breve*: the wild-type UCC2003 and its EPS-negative mutant (termed UCC2003_430), and strains NCIMB 702258, JCM 7017, and JCM 7019.

2. Materials and Methods

2.1. Bacterial growth, extraction and purification of PSs

B. breve strains were grown overnight in anaerobic conditions in modified de Man, Rogosa and Sharpe (MRS) medium containing 1% ribose (Merck, Germany), 100 μg/mL mupirocin (Biosynth, USA), 10 μg/mL tetracycline (where appropriate; Merck) and 0.05% L-cysteine hydrochloride (Merck).

Cells were collected by centrifugation, washed twice with deionized water and subjected to stepwise extractions by particular acids as described previously [30,31]. Briefly, cell pellets were resuspended in 5% trichloroacetic acid (TCA; Avantor, USA) and stirred for 48 h at 4 °C followed by centrifugation (12,000*g* x *g*, 10 min). The supernatant was dialyzed and freeze-dried (TCA extract). The pellet was resuspended in

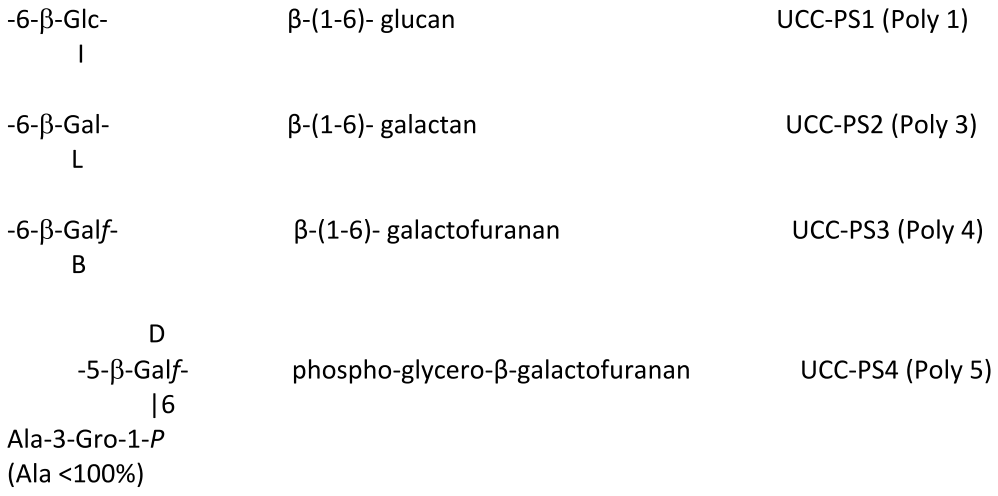


Fig. 2. Structural elements of cell-associated PSs isolated from the enzymatic extract of *B. breve* UCC_2003.

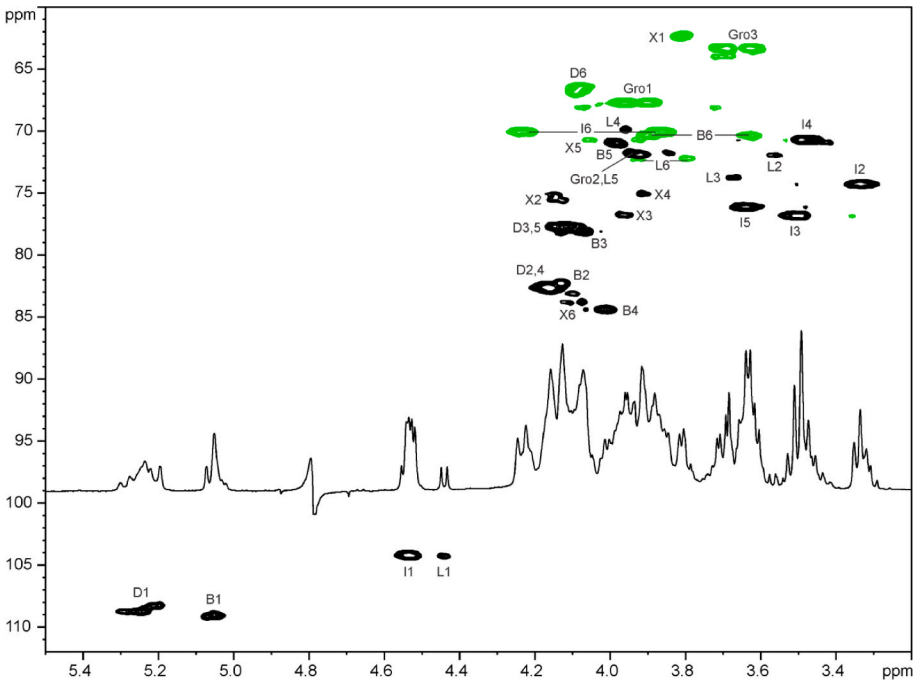


Fig. 3. $^{1}H–^{13}C$ HSQC spectrum of the *B. breve* UCC2003 EPS purified by anion-exchange chromatography.

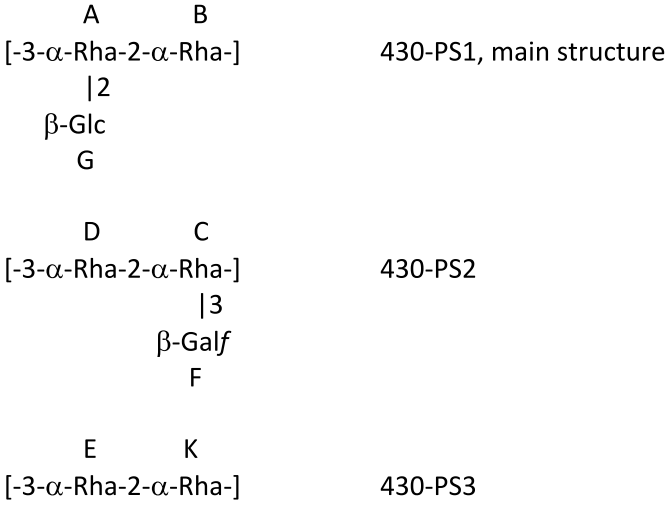


Fig. 4. Proposed structures of the repeating units of the CWPS from *B. breve* UCC2003_430.

0.01 M hydrochloric acid (HCl; Carlo Erba, Italy) extracted at 100 °C with stirring for 20 min, cooled, and centrifuged at 12,000*g* x *g*. This pellet was then re-extracted with 0.1 M HCl (Carlo Erba) in the same manner as for the 0.01 M HCl extraction. These two HCl extracts were deproteinized by adding TCA (Avantor) to a final concentration of 5%, dialyzed, and lyophilized to afford crude HCl extracts (0.01 M HCl and 0.1 M HCl; Carlo Erba).

Crude extracts were fractionated on a Sephadex G-50 column (Cytiva, UK), and the fractions were then screened by monosaccharide analysis and ^{1}H nuclear magnetic resonance (NMR) analysis. CWPSs were predominantly found in the 0.01 M HCl extracts. In *B. breve* 7019, preparations from 0.01 M HCl extract were used for structural analysis of PS1 and PS2. TCA and 0.1 M HCl extracts generally contained high molecular weight (HMW) glycogen (MW > 10 kDa), which is presumed to be due to contamination with intracellular content of lysed cells. A portion of cell pellet of *B. breve* JCM 7019 was freeze-dried and extracted with hydrofluoric acid (HF; 48%, 48 h at 5 °C; Acros Organics, Belgium). Following centrifugation, the extract was diluted with water, neutralized with potassium hydroxide (KOH; 8 M; Carlo Erba), and dialysed. TCA (up to 5%; Avantor) was added, the white precipitate removed by centrifugation, and the supernatant dialysed and freeze-dried. The HMW fraction of this extract was used for structural analysis of 7019-PS3.

For the initial CWPS characterisation of *B. breve* UCC2003 WT, cells were subjected to an enzymatic extraction essentially as described in 32. Briefly, a cell pellet of this strain (~15 g) was washed with water and resuspended in phosphate buffered saline (PBS; 100 ml; Merck), containing NaN_3 (20 mM; Merck), EDTA (5 mM; Acros Organics) and egg-white lysozyme (20 mg, Merck). The suspension was stirred at 5 °C for 48 h and centrifuged. The supernatant was deproteinated by addition of TCA (Avantor), dialysed, freeze-dried and fractionated on a Sephadex G-50 column (Cytiva). PSs eluted as three broad fractions, which were screened by monosaccharide analysis and ^{1}H NMR. The low molecular weight (LMW; <5 kDa) fraction contained mostly glycogen. The HMW fractions contained Glc and Gal in different ratios and had heterogeneous ^{1}H spectra. They were pooled and separated on an anion-exchange Hitrap Q (Cytiva) in several fractions which were analysed by NMR.

2.2. General and analytical methods

Chromatography, monosaccharide and methylation analyses, electrospray ionization mass spectrometry (ESI-MS) and NMR spectroscopy were performed as described previously [25,31]. Absolute L-configuration of 6dTal in the PS of *B. breve* JCM 7017 was determined by GC-MS of the acetylated (R)-(−)-2-butyl derivative by comparison with an authentic standard - OPS from *Pseudomonas* (*Burkholderia*) *plantarii* DSM 6535 was used as the source of D-6dTal [33].

For O-deacetylation, samples were treated with aqueous NH_4OH (12%, 100 °C, 30 min) and desalted on a Sephadex G-15 column (Cytiva).

Methylation was performed as reported previously [34]. 0.5 mg of a given PS (mixture) was dissolved in 0.5 mL of dry dimethyl sulfoxide (DMSO; Thermo Fisher Scientific, USA), powdered sodium hydroxide (NaOH; about 50 mg; Carlo Erba) was added and the resulting mixture

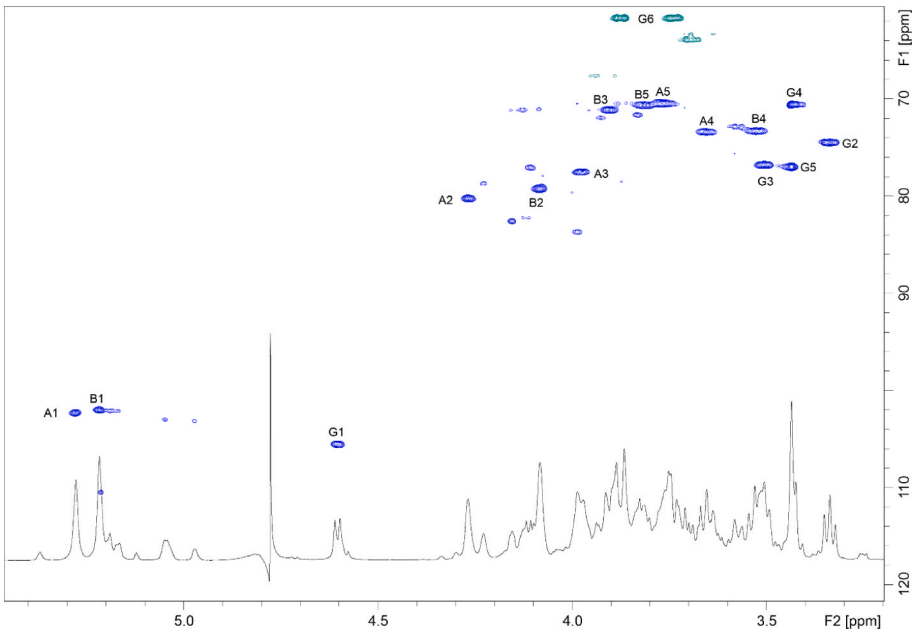


Fig. 5. ^{1}H–^{13}C HSQC spectrum of the *B. breve* UCC2003_430 CWPS showing 430-PS1 units (430-PS2 and 430-PS3 are present but not labeled due to the corresponding signals not being visible using the signal threshold presented above).

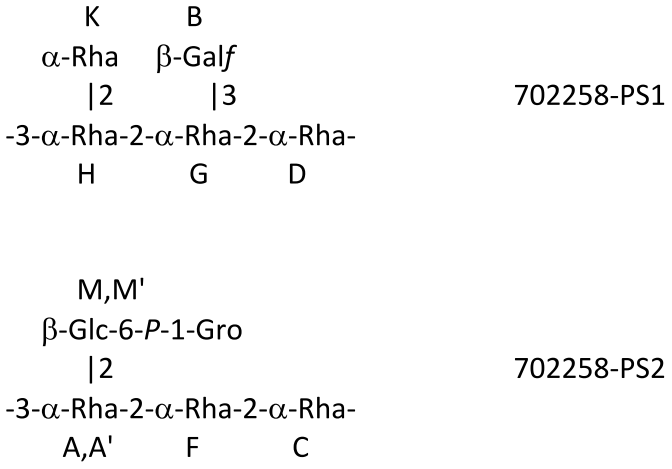


Fig. 6. Structures of CWPS from *B. breve* NCIMB 702258.

was stirred for 30 min, then 0.2 mL of methyl iodide (MeI; Merck) was added, followed by stirring of the mixture for 30 min, flushed with air to remove MeI, diluted to 10 mL with water, passed through C18 Sep-Pak cartridge (Waters Corporation, USA), washed with 10 mL of water and the methylated compound eluted with 5 mL of methanol. The product was hydrolysed with 3 M trifluoroacetic acid (TFA; 120 °C, 3 h; Merck), dried, reduced with $NaBD_4$ (Merck), reagent destroyed with 0.5 mL of 1 M HCl (Carlo Erba), solution dried under a stream of air, dried twice with addition of methanol (MeOH; 1 mL; Avantor), acetylated with 0.2 mL Ac_2O (Merck) - 0.2 mL pyridine (Acros Organics) for 30 min at 100 °C, dried, analysed by GC-MS on Thermo Trace 1310 instrument with ITQ1100 ion trap detector, capillary column HP-5 (Thermo Fisher Scientific), 160-260 °C by 4 °C/min.

3. Results and discussion

The main aim of our research efforts was to obtain insights into the diversity and structural nature of CWPSs produced by a number of *B. breve* strains. These strains were selected as they represented members of the same species, were relatively easy to grow and had been subjected to previous characterisations (e.g. genome sequencing, carbohydrate utilization analysis, and other investigations [15,18,35–37]).

We used an extraction method commonly employed for retrieval of CWPSs that are strongly (i.e. covalently) bound to the peptidoglycan (PG) layer [30], adapted essentially from that described by Prakobphol & Linzer [38]. The method is based on CWPS extraction by hot dilute HCl, following a pre-extraction step intended to remove other, less tightly bound surface PSs with cold 5% TCA (Fig. 1; Avantor).

3.1. *B. breve* UCC2003 WT

Crude 0.01 M and 0.1 M HCl extracts of *B. breve* UCC2003 contained variable amounts of Rha, Glc and Gal indicating the presence of a mixture of different CWPS and of other cell surface-associated PSs or their fragments. Purification of these components was unsuccessful, which may be due to a high degree of acid-mediated degradation (this was subsequently borne out in the cases of UCC-PS3 and UCC-PS4 (Fig. 2) which contain the particularly acid labile glycosidic linkage of Gal*f*). We therefore attempted a mild extraction method based on the enzymatic digestion of the cell wall. A *B. breve* UCC2003 cell pellet was treated with lysozyme in the presence of EDTA, as described in the Materials and Methods section, and the resulting crude extract was fractionated on a Sephadex G-50 column. PSs eluted as three broad fractions, which were assessed by monosaccharide analysis and ^{1}H NMR.

The HMW void volume fraction (Fraction 1, MW > 10 kDa), and the LMW fraction (Fraction 3) mostly contained a glycogen-type polymer (which we assumed was an intracellular bacterial glycogen [23,27]). Fractions with K_{av} ~0.1-0.5 (Fraction 2) contained Glc and Gal in different ratios and produced heterogeneous ^{1}H spectra. They were pooled and separated on an anion-exchange Hitrap Q in several fractions. Despite this separation attempt all obtained fractions appeared to represent PS mixtures. 2D NMR analysis allowed the elucidation of several polysaccharidic structures (Figs. 2 and 3, Supplementary Table S1). Most of these closely resemble glycans described previously for other strains of bifidobacteria [23], and in particular in *B. bifidum* strain PRI1 [39]. The labelling used in reference 39 is indicated in brackets for comparative purposes.

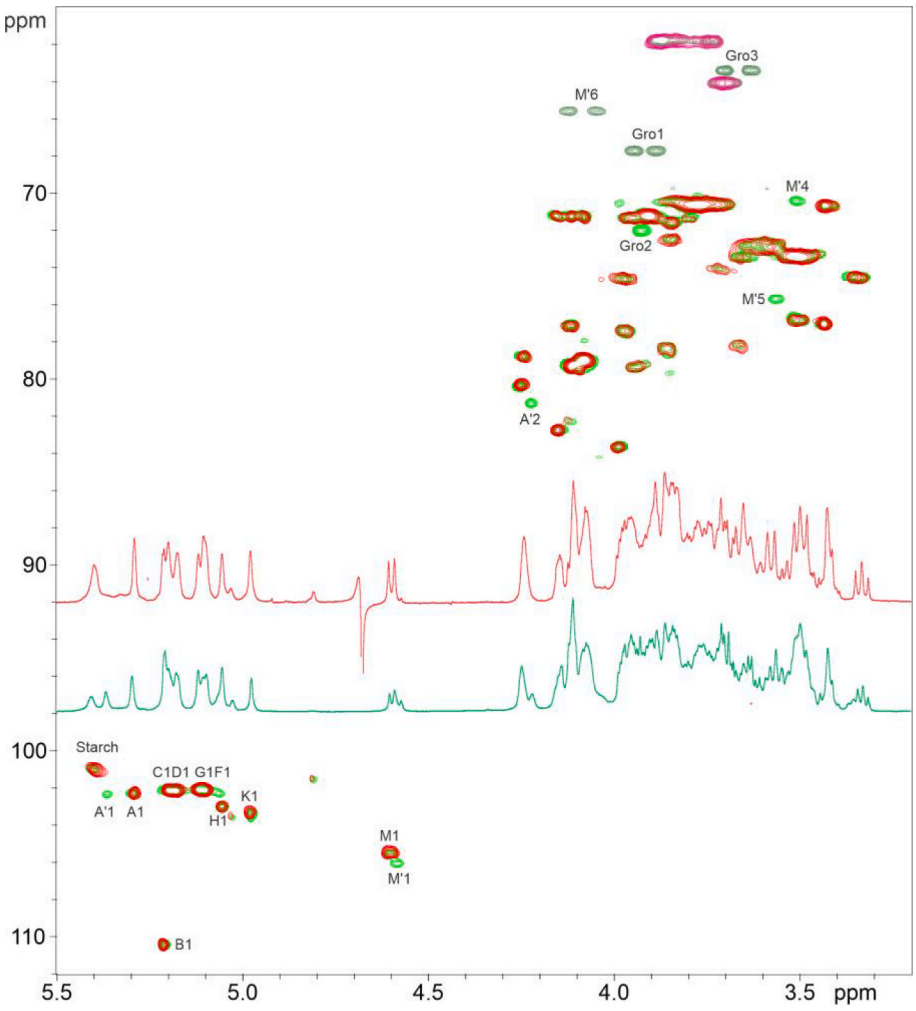


Fig. 7. Overlap of HSQC spectra of intact (green-dark green, ^{1}H green) and neutral fraction (Q1) (red-pink, ^{1}H red) PSs from *B. breve* NCIMB 702258.

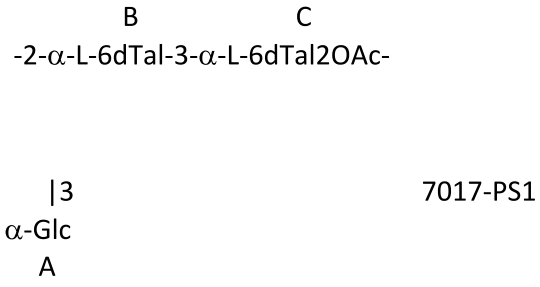


Fig. 8. Structure of the CWPS from *B. breve* JCM 7017.

The main differences between the structures described here and those obtained for *B. bifidum* as described in reference 39 are the absence of an α-Glc side chain in any of the PSs, alanine substitution on Gro, and absence of the β-(1-4)-galactan (therein termed Poly 2). Since all PSs elute as charged fractions, they are likely linked in some way to the phosphorylated UCC-PS4. Linear PSs, containing either β-(1,5)-linked Gal*f* groups (UCC-PS4) or β-(1,6)-linked Glc*p* groups (UCC-PS1), were previously identified as polysaccharide parts of LTA from *B. bifidum* spp. *pennsylvanicum* [40]. It is possible that also in *B. breve* UCC2003, these structural elements are associated with deacylated populations of LTA.

Preparations of Rha-containing CWPS from *B. breve* UCC2003 WT were found to be very heterogeneous and difficult to analyse due to the presence of various, interfering complex cell-surface associated polysaccharides described above. In order to allow proper interpretation of the obtained data without interference of EPS-derived signals we used a previously characterised EPS-negative isogenic mutant of this strain named *B. breve* UCC2003_430 [18] for CWPS studies, discussed below.

3.2. *B. breve UCC2003_430*

The 0.01 M HCl extract of *B. breve* UCC2003_430 cells contained Rha, Glc and Gal in an approximate ratio of 2.5: 1: 0.14. Methylation analysis revealed the presence of 2-, 3-, 2,3-linked Rha and terminal Glc. The CWPS was subjected to detailed 2D NMR analysis, allowing the complete assignment of its ^{1}H- and ^{13}C spectra (Supplementary Table S2). Analysis of the obtained NMR data led to the conclusion that the extract contained a CWPS with three main types of repeating units (430-PS1, 430-PS2 and 430-PS3; Figs. 4 and 5), differing by the type and position of monosaccharide substitution. The following NOE and HMBC cross peaks were observed: NOE. 430-PS1 A1:B2, B1:A3, B1:A5, G1:A2. 430-PS2 C1:D3, C1:D5, D1:C2, F1:C3. R3 E1:K2, K1:E3, K1:E5. HMBC. 430-PS1 A1:B2, B1:A3, G1:A2. 430-PS2 C1:D3, D1:C2, F1:C3. 430-PS3 E1: K2, K1:E3.

Units 430-PS1, 430-PS2 and 430-PS3 were shown to be present in blocks made of the same unit, long enough to look like regular PS (i.e. composed of repeating units). Linkages between different units are not visible yet may be present. Various small, unassigned rhamnose signals were present, possibly from the connections between different units.

The major structural element of the *B. breve* UCC2003_430 (430-PS1) has a close similarity to the previously identified "1,2/1,3 rhamnose

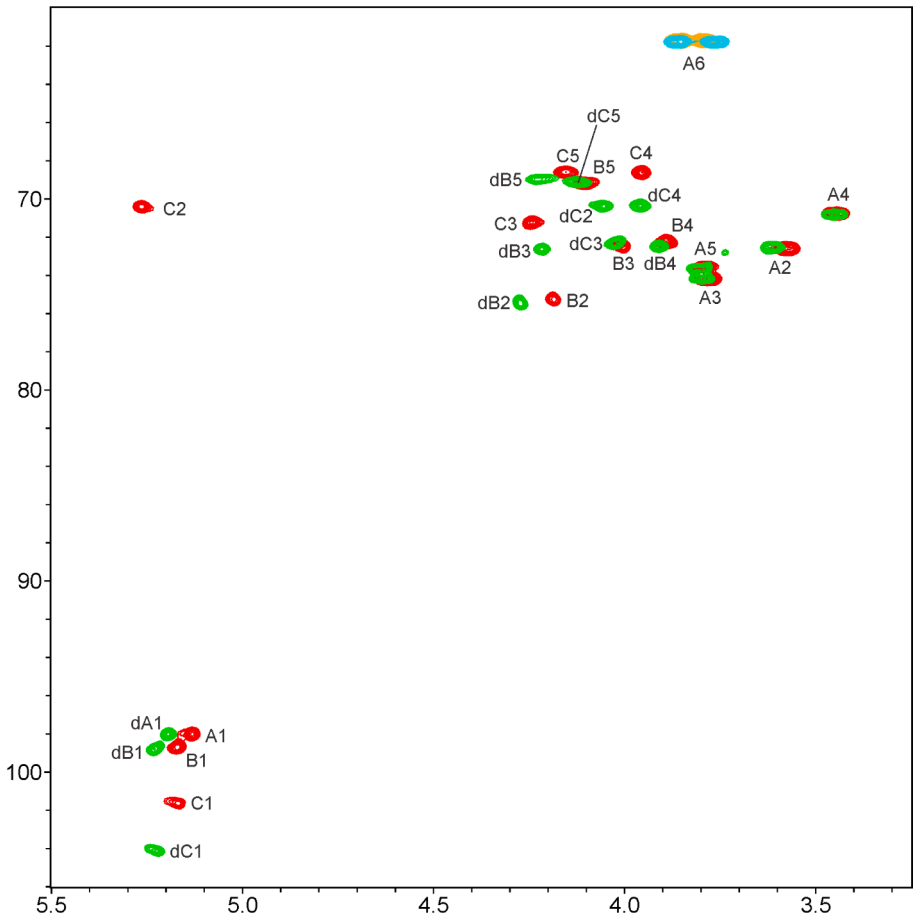


Fig. 9. HSQC spectrum of the CWPS (red-orange) and *O*-deacylated CWPS (DPS, green-cyan, signals labeled "d" except for Glc A) from *B. breve* JCM 7017.

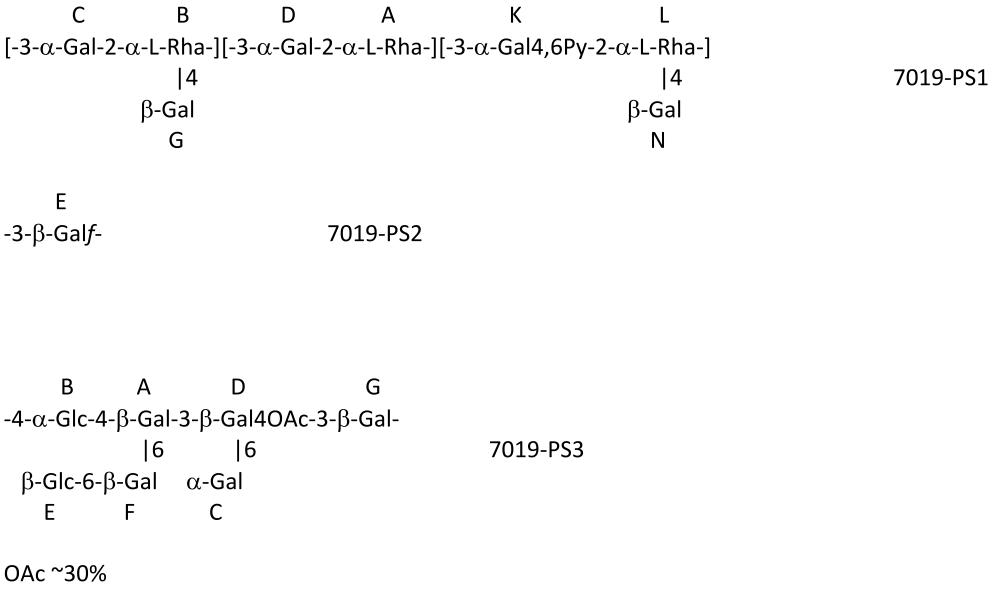


Fig. 10. Structures of the CWPSs from *B. breve* JCM 7019.

polymer" from *B. bifidum* ssp. *pennsylvanicum*, with the difference of β-Glc instead of α-Glc substitution of the 3-α-Rha-of the main backbone [27].

3.3. *B. breve NCIMB 702258*

Upon fractionation, the TCA and 0.1 M HCl extracts contained a mix of two major PS fractions: HMW glycogen, eluted in a void volume, and a broad PS fraction with K_{av} ~0.2-0.5 (designated here as $CWPS_{2258}$). The 0.01 M HCl extract largely contained apparent CWPS and was therefore

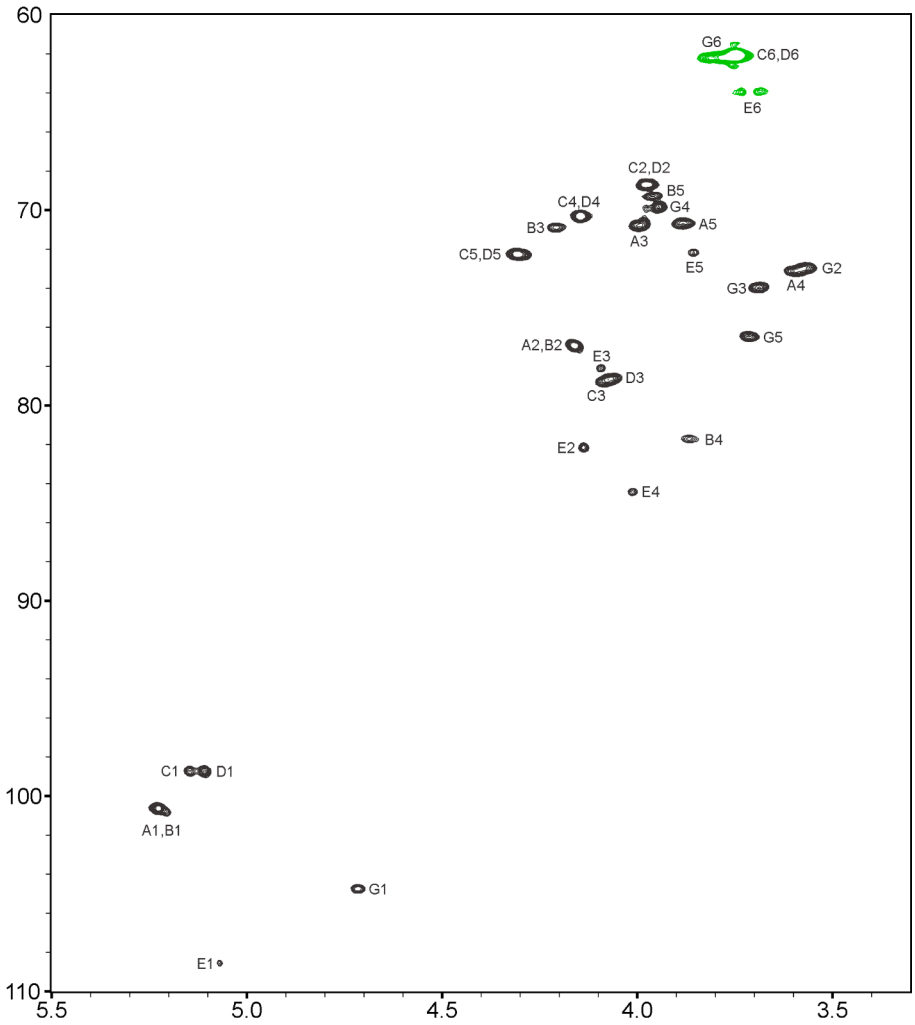


Fig. 11. HSQC spectrum of the mixture of 7019-PS1 and 7019-PS2 from *B. breve* JCM 7019.

used for further analysis. This extracted $CWPS_{2258}$ was shown to contain Rha, Glc and Gal in an approximate ratio of 3.3:1:0.5. Methylation analysis of $CWPS_{2258}$ showed the presence of 2-linked Rha, 3-linked Rha, 2,3-linked Rha, terminal Glc, terminal Gal, as well as 4–linked Glc residues. Original spectra of CWPS showed heterogeneity with several minor O-acetate signals being visible. The sample was de-*O*-acetylated, which did not improve the quality of NMR spectra. The resulting de-*O*-acetylated PS was separated on an anion-exchange column, giving three fractions: a neutral fraction and a wide fraction at the beginning of the gradient, collected in two parts (charged fraction 1 and charged fraction 2). All fractions contained the CWPS with different degrees of purity. The neutral fraction contained most of the glycogen contamination, charged fraction 1 was a mixture and charged fraction 2 was mostly free from glycogen and contained additional components compared to the neutral fraction. The signals from glycogen did not interfere with spectra of the presumed $CWPS_{2258}$.

Detailed NMR analysis of the neutral fraction and charged fraction 2 indicated that CWPS from *B. breve* NCIMB702258 was composed of a trisaccharide backbone (-3-α-Rha-2-α-Rha-2-α-Rha-) with two different types of side chains in an approximate ratio of 1:1 (702258-PS1 and 702258-PS2; Supplementary Table S3; Fig. 6; Fig. 7). An additional acidic component was identified as Gro-1-*P*- at O-6 of β-Glc M' (Fig. 6). Spectra contained several additional rhamnose spin systems, and minor β-Gal. Minor components are not listed in Table S3. It was not possible to conclude whether there is one PS present or a mixture of two or more independent structures.

3.4. *B. breve JCM 7017*

Similar to strain NCIMB 702258 above, TCA and 0.1 M HCl extracts of *B. breve* JCM 7017 contained a mixture of CWPS and glycogen, while the 0.01 M extract contained mostly CWPS, which was therefore used for subsequent structural analyses (designated here as 7017-PS1). The 0.01 M HCl extract was shown to contain dTal and Glc in an approximate ratio 1:1.3 and traces of Rha and Gal. Presence of L-dTal was confirmed by GC-MS compared to an authentic sample of D-dTal [33]. The 0.01 M HCl extract was used for detailed NMR analysis, which showed that 7017-PS1 is composed of a repeating trisaccharide unit containing α-dTal disaccharide backbone and α-Glc branches. The α-dTal residue C carried an OAc group at position 2 (close to 100%, Supplementary Table S4, Figs. 8 and 9). This structure is identical to that recently reported for *Pseudolactococcus laudensis* strain DSM 28961 [31].

3.5. *B. breve JCM 7019*

B. breve JCM 7019 cells were extracted with consecutive treatment with acids, as described above. All acidic extracts contained Rha, Glc and Gal in varying ratios. Similarly to the other *B. breve* strains assessed here, the majority of PS material (~75%) was extracted with 0.01 M HCl. All extracts were fractionated on a Sephadex G-50 column, and the obtained fractions were then screened by monosaccharide analysis and ^{1}H NMR. The HMW fractions of acid extracts contained only Glc and Gal. Surprisingly, Rha was detected only in lower MW fractions in relatively

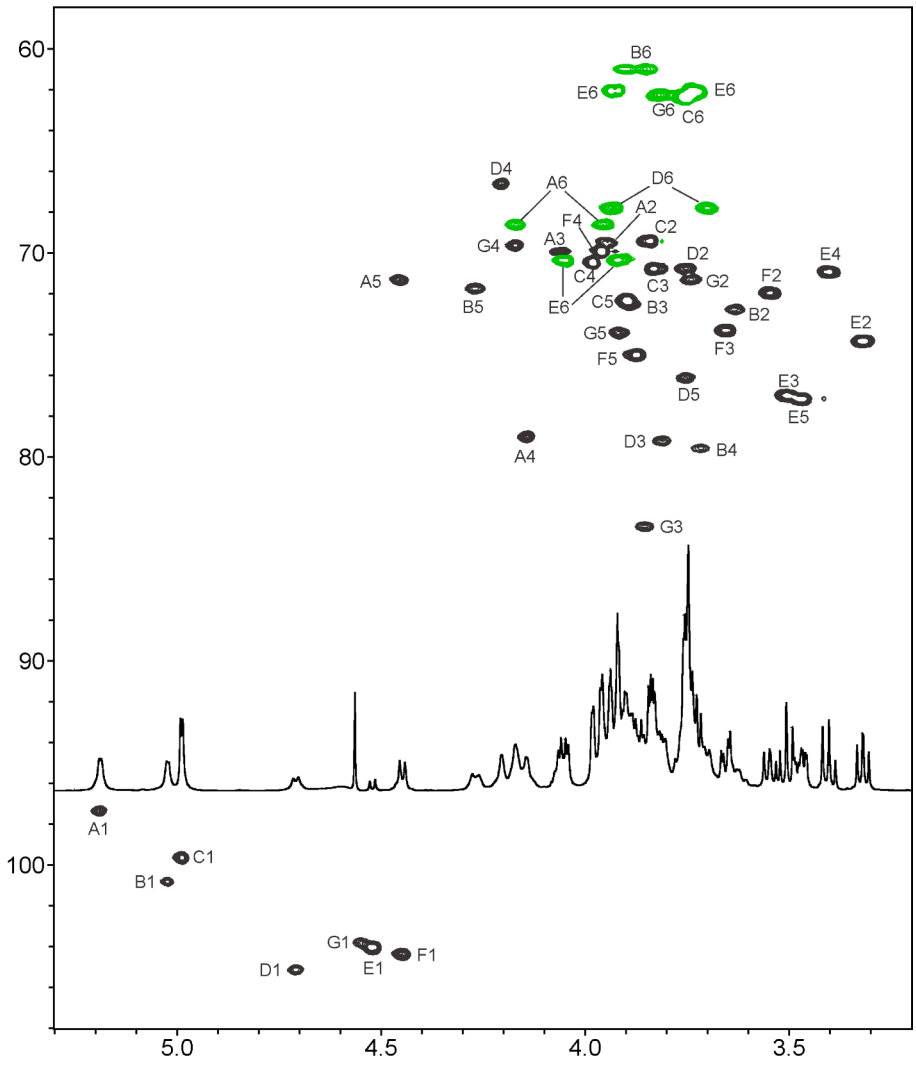


Fig. 12. Hsqc of the *O*-deacetylated 7019-PS3 from *B. breve* JCM 7019.

small amounts. We hypothesized that the rhamnose-rich CWPS of this strain, if present, was strongly bound to peptidoglycan. In an attempt to isolate the strongly bound CWPS, another portion of the cells was freeze dried, extracted with 48% HF (Acros Organics) and fractionated on a Sephadex G-50 column (Cytiva). Similarly to HMW fractions of HCl extracts, the HMW fraction of the HF extract contained only Glc and Gal in an approximate ratio 1:2.5. Analysis of different fractions allowed the identification of three distinct CWPSs termed 7019-PS1, 7019-PS2 and 7019-PS3 (Fig. 10).

Fractions with K_{av} ~0.2-0.5 of the 0.01 M HCl extract contained mostly Rha and Gal and were used for structural analysis of the mixture of 7019-PS1 and 7019-PS2 (Figs. 10 and 11, Supplementary Table S5). Interestingly, the structure of the [-3-α-Gal-2-α-L-Rha-] backbone of 7019-PS1 was identical to one previously found rhamnogalactan isolated from the cell wall of *B. longum* YIT4028 [41], differing only in side chain substitution. While the rhamnogalactan of *B. longum* YIT4028 had a pentasaccharide repeating unit, i.e. branched trisaccharide linked to the linear disaccharide; 7019-PS1 was shown to exhibit a ~1:2 ratio of branched to linear structures. In addition, a characteristic signal at 1.47/26.4 ppm indicated the presence of a pyruvate group. Indeed, upon further fractionation of the sample on an anion-exchange column, a negatively charged fraction was obtained enriched in pyruvate. Its analysis allowed the establishment of the position of the pyruvate group (Fig. 10). Pyruvate content was determined as 12% to all rhamnose by ^{1}H integral in the acidic fraction, thus the degree of substitution in 7019-PS1 was very small. The observed chemical shift of pyruvate C-3 at 26.4 ppm indicated its R-configuration [42].

The HMW fraction of the HF extract had homogeneous ^{1}H NMR spectra and was used for structural analysis of 7019-PS3. It was shown to be composed of branched heptasaccharide repeating unit, containing two Glc and five Gal*p* residues. (Figs. 10 and 12, Supplementary Table S6). The β-Gal*p* residue D carried an OAc group at position 4 (~30%). Methylation analysis of *O*-deacetylated PS3 showed the presence of terminal, 3-, 6-, 4,6-, and 3,6-linked Gal, as well as terminal and 4-linked Glc, in agreement with the established structure.

4. Conclusion

CWPSs from four strains of *B. breve* were (partially) purified and analysed. Their chemical structure was established by 2D NMR spectroscopy and methylation analysis. Each strain produced two or more polysaccharides, including (presumed intracellular) glycogen and CWPSs with common rhamnan substructures for strains UCC2003_430 and NCIMB 702258. The CWPS from *B. breve* JCM 7017 contained 6-d-Tal instead of Rha while the CWPS fraction of *B. breve* JCM 7019 contained three structurally distinct CWPSs. In addition, CWPS structures similar to those described for *B. bifidum* were identified for *B. breve* UCC2003. Our study represents the first detailed description of the chemical structures of PSs from the four strains of *B. breve*.

CRediT authorship contribution statement

Evgeny Vinogradov: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. **Irina Sadovskaya:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing – review & editing. **Brian McDonnell:** Investigation, Methodology, Writing – original draft, Writing – review & editing. **Keith Coughlan:** Investigation, Methodology, Writing – review & editing. **Francesca Bottacini:** Investigation, Methodology, Supervision. **Douwe van Sinderen:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Writing – original draft, Writing – review & editing.

Declaration of competing interest

Regarding the submitted manuscript 'Structural investigation of polysaccharides extracted from four *Bifidobacterium breve* strains' by Vinogradov et al., we the authors would like to confirm that we have no conflict of interest to declare.

Acknowledgements

D.V.S. is supported by Science Foundation Ireland (now Research Ireland) under grant numbers 12/RC/2273-P1 and 12/RC/2273-P2. Keith Coughlan is supported by MTU TU RISE Research Scholarships, co-financed by MTU and the Government of Ireland and the European Union through ERDF, Southern, Regional, Eastern & Midland Regional Programme 2021-27 and the Northern and Western programme 2021-27.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carres.2026.109946.

Data availability

Data will be made available on request.

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