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EXPERIMENTAL ARTICLES

Characterization of the Cell-Bound Polysaccharides of *Bifidobacterium adolescentis* 94 BIM

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Abstract—The cell-bound polysaccharides (PSs) of *Bifidobacterium adolescentis* 94 BIM were stripped from the cell surface with 2% sodium dodecyl sulfate (SDS), 1.5% Cetavlon, and 1% Triton X-100 and purified by precipitation with 5 volumes of ethanol. According to the extraction conditions used, the polysaccharide preparations were designated as PS-SDS-6°C, PS-SDS-100°C, PS-Cet, and PS-Trit. The gel-permeation chromatography of the first two preparations with the use of a Bio-Gel P-10 column and 1% acetic acid as the eluant yielded two peaks, F1 and F2, which contained carbohydrates and no phosphorus. All polysaccharides were primarily composed of glucose and galactose. The polysaccharides PS-Cet and PS-Trit were found to be branched and contain glucose residues at the terminal position, position 4, and position 6, and galactose residue at position 3. PS-SDS-6°C has a glucose residue at position 4.

Key words: bifidobacteria, polysaccharides.

Bifidobacteria are gram-positive, anaerobic, nonspore-forming bacteria inhabiting the gastrointestinal tract of humans and animals [1] and producing biologically active substances with immunomodulatory, radioprotective, and anticarcinogenic activities [2]. Bifidobacteria and lactic acid bacteria in situ reduce the risk of cancer owing to the inactivation of carcinogenic substances in the gastrointestinal tract, the inhibition of carcinogen-activating enzymes, and the stimulation of the immune system of organisms. The biological activity of bifidobacteria may be due to the action of substances constituting their cell walls, such as peptidoglycans, extracellular polysaccharides, glycoproteins, phospho- and glycolipids, and protein–lipoteichoic acid complexes [2, 3]. For instance, Sekine *et al*. showed that the polysaccharide components of the cell wall of some bifidobacteria possess immunostimulating activity and activity against murine sarcoma [4].

Our earlier studies dealt with the minute structure of the polysaccharide capsule of *Bifidobacterium bifidum* and *B. adolescentis* and their intercellular macromolecular matrix [5–7]. The protein–polysaccharide complex of *B. adolescentis* 94 BIM was found to contain proteins and carbohydrates in a ratio of 1 : 1. This complex stimulated bifidobacterial growth, acid formation, synthesis of extracellular enzymes and proteins, and the utilization of sugars by the physiologically active and dormant forms of bifidobacteria. The protein–polysaccharide complex of bifidobacteria is nontoxic to the *Tetrahymena pyriformis* W population and does not possess mutagenic and sensibilizing activities. At the same time, it exhibits high adaptogenic and proliferative activities [8, 9].

The aim of the present work was to isolate, purify, fractionate, and determine the chemical composition of polysaccharides associated with the cell wall of *B. adolescentis* 94 BIM.

MATERIALS AND METHODS

The strain *Bifidobacterium adolescentis* 94 BIM was derived from *B. adolescentis* MC-42 through *B. adolescentis* 91 BIM by means of selection with respect to antibiotic resistance and proteolytic activity. This strain is deposited in the collection of microorganisms at the Institute of Microbiology, National Academy of Sciences of Belarus, as *B. adolescentis* BIM B87.

The strain was cultivated at 37°C in a modified corn–lactose medium (CLM) [10] supplemented with casein hydrolysate and yeast extract. The medium was inoculated with physiologically active bifidobacterial cells of the third generation (18 h of growth). The inoculum size was 5 vol %. To study growth dynamics, acid formation, and the accumulation of end metabolic products in a bifidobacterial culture, it was sampled at 6-h intervals for 2 days. Growth was monitored by determining the dry weight of the biomass (for this purpose, cells were dried to a constant weight at 105°C) or by measuring the culture turbidity at 590 nm. The number of viable bifidobacterial cells was determined by plating culture dilutions onto agar media. The results were expressed in colony-forming units (CFU) per mil-

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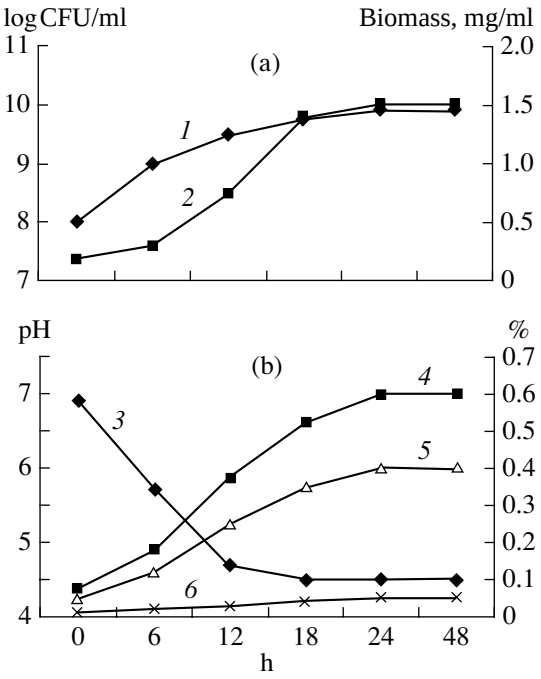

Fig. 1. (a) Growth of *B. adolescentis* 94 BIM in a modified corn–lactose medium expressed in (*1*) log CFU/ml and (*2*) mg dry cells/ml and (b) intensity of acid formation expressed in (*3*) pH and accumulation of (*4*) acetate, (*5*) lactate, and (*6*) ethanol.

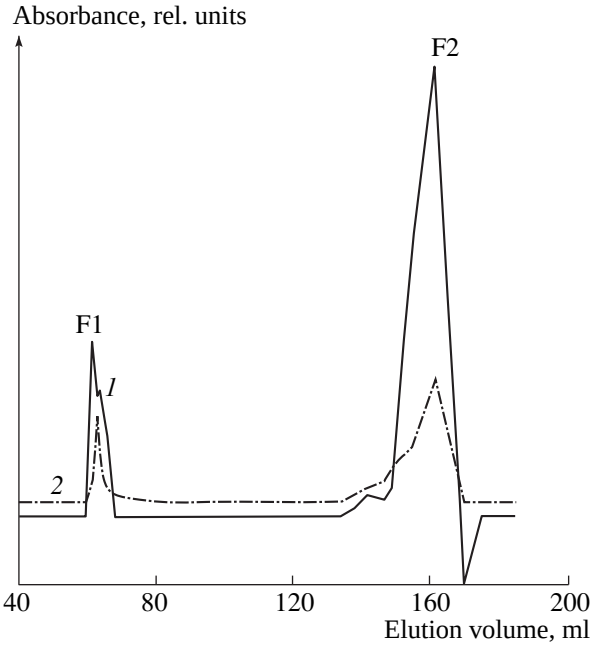

Fig. 2. Gel-permeation chromatography of PS-SDS-100°C on a column with Bio-Gel P-10 using 1% acetic acid as the eluant: (*1*) eluate absorbance and (*2*) the content of carbohydrates in the eluate, A_{490}.

liliter. The kinetic parameters of growth ($\log X$, μ, ν, g, where X is the biomass; the specific growth rate μ; the rate constant of cell division, v; and the generation time g) were calculated as described by Varfolomeev [11]. The content of acetic acid and ethanol was determined using a Chrom-5 gas chromatograph (Laboratorni pristroje, Czech Republic). The concentration of lactic acid was calculated from the glucose fermentation balance [12]. Cell-associated polysaccharides were isolated from a 24-h-old *B. adolescentis* 94 BIM culture with the following characteristics: the concentration of viable cells ~9.0×10^9 CFU/ml; biomass ~1.5 mg dry wt/ml; $\log X = 2.69$; $\mu = 0.54$ h^{-1}; $v = 0.77$; $g = 1.29$; pH 4.5; the concentration of acetate, lactate, and ethanol ~0.6, 0.4, and 0.05 wt %, respectively (Fig. 1). To prepare PSs, cells were washed with distilled water and extracted with 2% sodium dodecyl sulfate (SDS) at 6°C for 18 h or at 100°C for 30 min. PSs were precipitated from the extracts with 5 volumes of absolute ethanol, treated with nucleases, and subjected to ultrafiltration and lyophilization. The resultant polysaccharide preparations were designated as PS-SDS-6°C and PS-SDS-100°C. Alternatively, washed cells were resuspended in phosphate-buffered saline (PBS) containing 0.15 M NaCl in 10 mM phosphate buffer (pH 7.3) and sonicated for a total of 120 s in four 30-s bursts. The homogenate was centrifuged twice. The supernatant was dialyzed, treated with 1.5% Cetavlon or 1% Triton X-100, and centrifuged. PSs were precipitated from the supernatant with 5 volumes of ethanol, treated with nucleases, and lyophilized. The polysaccharides that were obtained with the use of Cetavlon and Triton X-100 were designated as PS-Cet and PS-Trit, respectively.

Polysaccharide preparations were subjected to gel-permeation chromatography on a column (2.5×95 cm) with Bio-Gel P-10 using 1% acetic acid as the eluant. Eluate fractions 2.2 ml in volume were analyzed for the presence of phosphorus and carbohydrates. Carbohydrate-containing fractions were pooled and lyophilized. Lyophilized material was hydrolyzed in 1 M HCl at 100°C for 4 h, and the hydrolysate was analyzed for monosugars by combined gas–liquid chromatography and mass spectrometry (GLC–MS) using a Hewlett-Packard HP5971A (series II) chromatograph–mass spectrometer equipped with an HP-1 column (0.2 mm × 12 m). During separation, the column temperature was raised from 150 to 270°C at a rate of 8°C/min.

The sugar analysis of PSs via permethylation [13] was carried out as described earlier [14, 15]. The content of carbohydrates, phosphorus, sialic and glucuronic acids, as well as the stereoconfiguration of monosugars in PSs were determined by enzymatic methods [14, 15].

RESULTS AND DISCUSSION

Analysis of polysaccharide preparations showed that their purity depended on the extraction temperature and the detergent used. When PSs were extracted from

Table 1. The carbohydrate composition of the cell-associated polysaccharides of *B. adolescentis* 94 BIM

Carbohydrate	Molar proportion of particular carbohydrate							
	PS-SDS-100°C			PS-SDS-6°C			PS-Cet	PS-Trit
	PS	F1	F2	PS	F1	F2		
Glucose	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Galactose	1.7	1.6	1.4	0.8	0.5	1.4	0.5	0.3
Mannose	0	0.5	0.6	0	0	1.8	0	0
Ribose	1.8	0	0.1	0.2	0	3.2	0	0
Glucosamine	0	0	0.1	0	0	0	0	0

B. adolescentis 94 BIM cells with the use of SDS, the PS preparation contained protein impurities of up to 50%. The gel-permeation chromatography of PS-SDS-6°C and PS-SDS-100°C samples (30 mg) on Bio-Gel P-10 using 1% acetic acid as the eluant yielded two peaks, F1 and F2, which contained carbohydrates and no phosphorus (Fig. 2).

The carbohydrates of crude PSs were represented by glucose, galactose, and ribose in a ratio of 1.0 : 1.7 : 1.8 (PS-SDS-100°C) and 1.0 : 0.8 : 0.2 (PS-SDS-6°C). The F1 and F2 fractions of PS-SDS-100°C contained glucose and galactose in ratios of, respectively, 1.0 : 1.6 and 1.0 : 1.4, as well as mannose. In addition, the fraction F2 of PS-SDS-100°C contained ribose and glucosamine. The F1 and F2 fractions of PS-SDS-6°C contained glucose and galactose in approximate ratios of 1.0 : 0.5 and 1.0 : 1.4, respectively. In addition, the fraction F2 of PS-SDS-6°C contained ribose and mannose. The presence of ribose in the PS preparations suggests that SDS impaired the integrity of the cell wall of bifidobacteria in the process of extraction, which resulted in the PS being contaminated with the degradation products of nucleic acids, ribose in particular.

At the same time, the extraction of PSs from *B. adolescentis* 94 BIM cells with the use of ultrasonic treatment and Cetavlon or Triton X-100 allowed us to obtain PSs which contained only glucose and galactose in an approximate molar ratio of 1.0 : 0.5 (PS-Cet) and 1.0 : 0.3 (PS-Trit). Analysis with the use of D-glucose oxidase, D-galactose oxidase, and peroxidase showed that PS-Cet and PS-Trit contain glucose in the D configuration. These PSs were also found to contain phosphorus and glucuronic acid in trace amounts and no sialic acid (data not presented).

The results of analysis of permethylated PSs by combined gas–liquid chromatography and mass spectrometry are presented in Table 2. Figure 3 exemplifies the GLC–MS spectra of PS-Cet and PS-Trit, which exhibit the presence of 2,3,4,6-tetra-*O*-methyl-glucose, 2,3,6-tri-*O*-methyl-glucose, 2,4,6-tri-*O*-methyl-galactose, and 2,3-di-*O*-methyl-glucose. The permethylation of PS-SDS-^{6}C yielded 2,3,6-tri-*O*-methyl-glucose. PS-Cet and PS-Trit were found to be branched and contain glucose residues at the terminal position, position 4, and position 6, and galactose residue at position 3. PS-SDS-6°C has glucose residue at position 4.

It was of interest to compare *B. adolescentis* 94 BIM polysaccharides with the bifidobacterial polysaccharides whose chemical composition and structure have been reported in the literature [16–20]. Nearly all polysaccharides of *B. bifidum* subsp. *pennsylvanicum*, *B. bifidum* YIT 4007, *B. breve* YIT 4010, *B. infantis* YIT 4025, *B. infantis* Reuter ATCC 15697, *B. longum* ATCC 15707, *B. longum* YIT 4028, and *B. adolescentis* YIT 4011 are neutral and contain hexoses and deoxyhexoses. It is believed that they are peptidoglycan derivatives. In chemical composition, the extracellular polysaccharides of bifidobacteria and lactic acid bacteria can be divided into homo- and heteropolysaccharides. The homopolysaccharides of lactic acid bacteria include the D-glucans (dextrans) of *Leuconostoc mesenteroides* and mutants of *Streptococcus mutants* and *S. sobrinus*, the β-D-glucans of *Pediococcus* spp. and *S. sobrinus*, fructans (such as the levan of *S. salivarius*), and other polygalactan-like polysaccharides. The known heteropolysaccharides of lactic acid bacteria include those which are produced by the meso- and thermophilic strains of *Lactococcus lactis, Lactobacillus casei, Lb. sake, Lb. rhamnosis, Lb. acidophilus, Lb. delbrueckii, Lb. helveticus*, and *S. thermophilus* [21]. The heteropolysaccharides of two *B. adolescentis* strains, YIT 4011 and M101-4, were found to contain glucose and 6-deoxytalose with a small amount of gly-

Table 2. The permethylation analysis of the cell-associated polysaccharides of *B. adolescentis* 94 BIM

Methylated carbohydrate	Molar proportion of particular methylated carbohydrate			
	PS-SDS-6°C		PS-Cet	PS-Trit
	F1	F2		
2,3,4,6-tetra-*O*-methyl-glucose	–	–	1.0	1.0
2,3,6-tri-*O*-methyl-glucose	1.0	1.0	2.5	3.2
2,4,6-tri-*O*-methyl-galactose	–	–	0.9	1.0
2,3-di-*O*-methyl-glucose	–	–	0.9	1.3

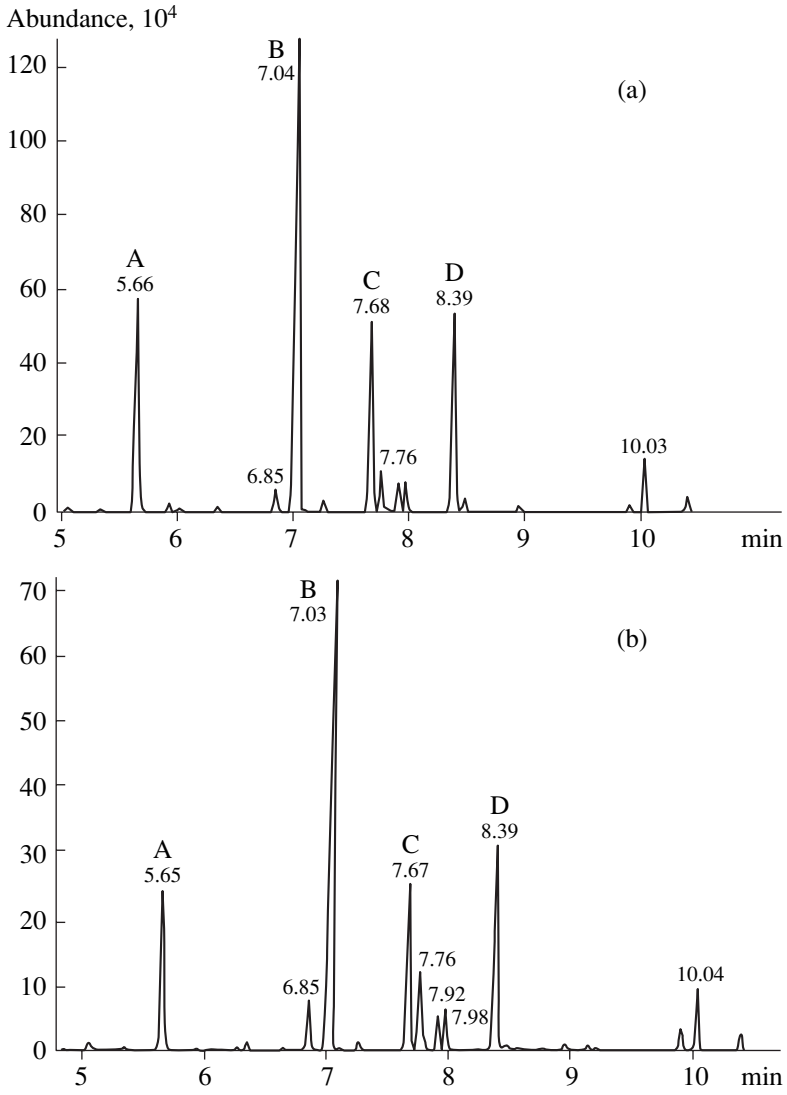

Fig. 3. The GLC–MS analysis of the permethylated (a) PS-Cet and (b) PS-Trit: (A) 2,3,4,6-tetra-*O*-methyl-glucose, (B) 2,3,6-tri-*O*-methyl-glucose, (C) 2,4,6-tri-*O*-methyl-galactose, and (D) 2,3-di-*O*-methyl-glucose.

copeptides (YIT 4011) and glucose and galactose (M101-4) [3, 19]. It can be seen than these polysaccharides differ from the polysaccharides of *B. adolescentis* 94 BIM under study.

The biologically active heteropolysaccharides of bifidobacteria and lactic acid bacteria, which reduce the level of cholesterol in the blood and possess anticarcinogenic and immunomodulating activities, are presently used in the production of therapeutic and prophylactic agents, yogurts, and nutriceutics [21]. As for their role for the producers, these heteropolysaccharides, together with glycoproteins, protect bifidobacterial populations from various unfavorable endogenous and exogenous factors. In the exponential growth phase, bifidobacteria secrete capsular polymers, while they produce a hypertrophic intercellular matrix in the stationary and death growth phases [6]. The build-up of capsular polysaccharides promotes the aggregation of dividing cells into discrete microcolonies and highly organized populational colonies of a mycelial type, whose integrity is maintained by multiple intercellular bonds [6, 7]. The crucial role in cell aggregation is played by polysaccharides and glycoproteins. The formation of multicellular structures enhances the adaptive and physiological stability of bifidobacterial populations [6, 7]. It should be noted that the extracellular polysaccharides and glycoproteins of *B. adolescentis* 94 BIM possess high bifidogenic activity and promote the adaptation and growth of the *Tetrahymena pyriformis* W population [8, 9].

We believe that further investigations of bifidobacterial polysaccharides and glycoproteins with respect to their chemical composition, structure, and biological activity may greatly contribute to the development of new efficient therapeutic, prophylactic, and biologically active agents.

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REFERENCES

1. Biavati, B., Sgorbati, B., and Scardovi, V., The Genus *Bifidobacterium, The Prokaryotes*, Balows, A., Ed., New York: Springer-Verlag, 1992, vol. 1, pp. 816–833.
2. Chesheva, V.V. and Manvelova, M.A., Anticarcinogenic and Immunomodulatory Activities of Bifidobacteria, *Meditsinskie aspekty mikrobnoi ekologii* (Medical Aspects of Microbial Ecology), Moscow, 1994, pp. 82–86.
3. Hosono, A., Lee, J., Ametani, A., Natsume, M., Hirayama, M., Adachi, T., and Kaminogawa, S., Characterization of a Water-Soluble Polysaccharide Fraction with Immunopotentiating Activity from *Bifidobacterium adolescentis* M101-4, *Biosci. Biotechnol. Biochem.*, 1997, vol. 61, pp. 312–316.
4. Sekine, K., Toida, T., Saito, M., Kuboyama, M., Kawashima, T., and Hashimoto, Y., A New Morphologically Characterized Cell Wall Preparation (Whole Peptidoglycan) from *Bifidobacterium infantis* with a Higher Efficacy on the Regression of an Established Tumor in Mice, *Cancer Res.*, 1985, vol. 45, pp. 1300–1307.
5. Novik, G.I., Vysotskii, V.V., and Bogdanovskaya, Zh.N., Cell Ultrastructure of Some *Bifidobacterium* Species, *Mikrobiologiya*, 1994, vol. 63, no. 3, pp. 515–522.
6. Novik, G.I. and Vysotskii, V.V., Architectonics of Bifidobacterial Populations: The Cohesion of *Bifidobacterium adolescentis* and *Bifidobacterium bifidum* Cells on a Submicroscopic Scale, *Mikrobiologiya*, 1995, vol. 64, no. 2, pp. 222–227.
7. Novik, G.I., Investigation of the Structural and Functional Organization of Bifidobacteria, *Mikrobiologiya*, 1998, vol. 67, no. 3, pp. 376–383.
8. Novik, G.I., Astapovich, N.I., Samartsev, A.A., and Ryabaya, N.E., Isolation and Characterization of the Extracellular Protein–Polysaccharide Complex of *Bifidobacterium adolescentis*, *Mikrobiologiya*, 1997, vol. 66, no. 5, pp. 621–627.
9. Novik, G.I., Astapovich, N.I., Ryabaya, N.E., and Bogdan, A.S., Investigation of the Biological Activity of the Extracellular Protein–Polysaccharide Complex of *Bifidobacterium adolescentis*, *Mikrobiologiya*, 1997, vol. 66, no. 5, pp. 628–634.
10. Sundukova, M.B., Khor'kova, E.A., Semenikhina, V.F., Ganina, V.I., Khaleneva, M.P., Pospelova, V.V., and Rakhimova, N.G., *Nutrient Medium for the Isolation and Cultivation of Bifidobacteria*, USSR Inventor's Certificate 3794941/30-13, *Byull. Izobret.*, 1986, no. 43.
11. Varfolomeev, S.D., Kinetics of Developing Microbial Populations, *Sovremennye problemy biokinetiki* (Modern Problems in Biokinetics), Varfolomeev, S.D., Ed., Moscow: Mosk. Gos. Univ., 1987, pp. 6–77.
12. Lauer, E. and Kandler, O., Mechanismus der Variation des Verhalthisses Acetat Lactat bei der Vergarung von Glucose durch Bifidobacterien, *Arch. Microbiol.*, 1976, vol. 110, pp. 271–277.
13. Ciucanu, I. and Kerek, F., A Simple and Rapid Method for the Permethylation of Carbohydrates, *Carbohydr. Res.*, 1984, vol. 131, pp. 209–217.
14. Gamian, A., Katzenellenbogen, E., Romanowska, E., Fernandez, J.M.G., Pedersen, C., Ulrich, J., and Defaye, J., Structure of the *Hafnia alvei* Strain PCM 1188 *O*-Specific Polysaccharide, *Carbohydr. Res.*, 1995, vol. 277, pp. 245–255.
15. Gamian, A., Katzenellenbogen, E., Romanowska, E., Dabrowski, U., and Dabrowski, J., Lipopolysaccharide Core Region of *Hafnia alvei*: Structure Elucidation Using Chemical Methods, Gas Chromatography–Mass Spectrometry, and NMR Spectroscopy, *Carbohydr. Res.*, 1995, vol. 266, pp. 221–228.
16. Tone-Shimokawa, Y., Toida, T., and Kawashima, T., Isolation and Structural Analysis of Polysaccharide Containing Galactofuranose from the Cell Walls of *Bifidobacterium infantis*, *J. Bacteriol.*, 1996, vol. 178, pp. 317–320.
17. Veerkamp, J.H., Hoelen, G.E.J.M., and Op Den Camp, H.J.M., The Structure of a Mannitol Teichoic Acid from *Bifidobacterium bifidum* subsp. *pennsylvanicum*, *Biochim. Biophys. Acta*, 1983, vol. 755, pp. 439–451.
18. Habu, Y., Nagaoka, M., Yokokura, T., and Azuma, I., Structural Studies of Cell Wall Polysaccharides from *Bifidobacterium breve* YIT 4010 and Related *Bifidobacterium* Species, *J. Biochem.*, 1987, vol. 102, pp. 1423–1432.
19. Nagaoka, M., Muto, M., Yokokura, T., and Mutai, M., Structure of 6-Deoxytalose-containing Polysaccharide from the Cell Wall of *Bifidobacterium adolescentis*, *J. Biochem.*, 1988, vol. 103, pp. 618–621.
20. Nagaoka, M., Shibata, H., Kimura, I., Hashimoto, S., Kimura, K., Sawada, H., and Yokokura, T., Structural Studies on a Cell Wall Polysaccharide from *Bifidobacterium longum* YIT 4028, *Carbohydr. Res.*, 1995, vol. 274, pp. 245–249.
21. Vuyst, L.D. and Degeest, B., Heteropolysaccharides from Lactic Acid Bacteria, *FEMS Microbiol. Rev.*, 1999, vol. 23, pp. 153–177.