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EXPERIMENTAL ARTICLES

Analysis of Phospholipids in Bifidobacteria

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Abstract—Methods of preparative chromatography on silica gel columns were used for obtaining preparations of polar lipids of bifidobacteria. Studies of the preparations by one-dimensional and two-dimensional TLC demonstrated that diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and phosphatidylcholine (PC) were the predominant phospholipids; minor phospholipids (phosphorus-containing components present in considerably lower amounts) included phosphatidylinositol (PI) and lyso-phosphatidylcholine (lyso-PC). Parameters of qualitative composition of phospholipids and glycolipids may serve as a set of chemotaxonomic markers in modern procedures for identifying *Bifidobacterium* species.

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Microorganisms of the genus *Bifidobacterium*—symbionts of the gastrointestinal tract of humans and animals—have been separated into an individual taxonomic unit. The questions of the position of bifidobacteria (within the kingdoms of microorganisms), their taxonomy, species differentiation, importance in medicine (treatment and prevention of diseases), and immunological implications had been the subject of debate for many years until the genus *Bifidobacterium* was identified as an autonomous taxonomic unit in 1924 (Orla-Jensen); later, it was classified with the family Actinomycetaceae of the order Acinomycetales (Bergey's Manual, 8th edition) [1, 2].

Reports on the taxonomy of bifidobacteria (including those describing the development of molecular-genetic methods of study) are numerous. Nevertheless, primary classification of natural isolates and differentiation of components of biological material (obtained from patients with diverse types of mixed infections caused by opportunistic flora) is highly problematic due to similarity of phenotypic indices of microorganisms. From this standpoint, analytical methods forming the base of chemotaxonomy can be used for identifying bifidobacteria and diagnosing certain diseases [3, 4].

Phospholipids, together with fatty acids and ubiquinones, are the most informative chemotaxonomic markers. For example, representatives of the family Nocardiaceae (comprising the genera *Corynebacterium*, *Gordona*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, and *Tsukamurella*) are characterized by type IV-A cell walls and type II phospholipids (the presence of diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), PI mannosides; the absence of phosphatidylcholine (PC) and glucosamine-containing phospholipids). For *Bifidobacterium* spp. and *Lactobacillus* spp., the differences in the composition of phospholipids (particularly polyglycerol phospholipids and aminoacyl phospatidylglycerols) is a strong argument in favor of chemotaxonomic differentiation of these microorganisms at the genera level [4–6].

The goal of this work was to isolate and analyze phospholipids of bifidobacteria.

MATERIALS AND METHODS

The objects our studies were bifidobacterium strains *Bifidobacterium longum* B 379 M, *B. bifidum* 791 (courtesy of researchers of the Gabrichevsky Research Institute of Epidemiology and Microbiology, Moscow), and *B. adolescentis* 94 BIM (deposited—as *Bifidobacterium adolescentis* BIM B-87—with the Scientific Collection of Type and Industrially Important Nonpathogenic Microorganisms of the Institute of Microbiology, National Academy of Sciences of Belarus; known as the Belarussian Collection of Nonpathogenic Microorganisms).

The microorganisms were cultured in a modified synthetic Eagle's medium for tissue culture (supplemented with 0.5% lactose and 0.05% ascorbic acid) and TSB medium. A physiologically active culture of bifi-

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† Deceased.

Table 1. Analysis of phosphorus-containing lipid components of *B. adolescentis* 94 BIM, as determined by TLC

Fraction	Phospholipid							
	DPG	PG	PI	PE	PC	Lyso-PC	PA	Lyso-PS
Lipid extract	+	+	+	+	+	+	+	+
Column I								
I-Met	–	–	–	–	–	–	+	–
II-Met	–	–	+	–	–	–	+	–
III-Met	–	+	+	–	–	+	–	–
IV-Met	–	+	+	–	–	+	–	–
Column II								
I-5% Met	–	–	–	–	–	–	–	–
II-5% Met	–	–	–	–	–	–	–	–
I-10% Met	–	–	+	–	+	–	–	–
IV-10% Met	–	–	–	–	–	+	–	+
III-15% Met	–	–	–	–	–	+	–	–
III-20% Met	–	–	–	–	–	–	–	–
I-30% Met	–	–	–	–	–	+	–	+

Note: (+) and (–) designate, respectively, positive and negative reactions with molybdenum reagent.

Table 2. Identification of dominant phospholipids in bifidobacteria

Strain	Phospholipid							
	DPG	PG	PI	PE	PC	Lyso-PC	PA	Lyso-PS
B. adolescentis 94 BIM	+++	+++	++	+++	+++	++	+	+
B. bifidum No. 791	+++	+++	++	+++	+++	++	+	+
B. longum B 379 M	+++	+++	++	+++	+++	++	+	+

Note: (+++), (++), and (+) designate, respectively, the pronounced, moderate, and weak positive reactions with molybdenum reagent.

dobacteria (generation III), grown on TSB medium, served as the inoculum. Bifidobacteria were grown under microaerophilic conditions at 37°C (thermostated chamber) for 24–48 h (steady-state growth phase). Bifidobacteria were separated from the culture liquid by centrifugation (36 000 *g*) on a Heraeus Sepatech Biofuge 28 RS (Germany). The cells were killed by treatment with 1% formalin (v/v) and washed three times by phosphate-buffered saline (PBS; 0.01 M H_3PO_4, 0.15 M NaCl, pH 7.3) to eliminate components of the medium and residual formalin. Extraction of the lipid component from the cells of bifidobacteria and subsequent isolation of glycolipid and phospholipid fractions were performed as described in our preceding reports [7, 8].

The lipid component thus obtained was subjected to preparative column chromatography. Column I was packed with Silica gel 60 (70–230 mesh; Merck). Lipids were eluted by chloroform, acetone, and methanol. Lipid fractions were designated with account for the eluant type and volume: I-Chl–IV-Chl (chloroform), I-Ac–V-Ac (acetone), and I-Met–IV-Met (methanol). The fraction I-Ac (122.5 mg) containing glycolipids was further separated by preparative column chromatography. Column II was packed with Silica gel 60 (200–300 mesh; Merck) and eluted with a gradient of methanol in chloroform. The following designations were used: I-Chl2 and II-Chl2; I-5% Met–IV-5% Met; I-10% Met–IV-10% Met; I-15% Met–IV-15% Met; I-20% Met–III-20% Met; I-30% Met and II-30% Met; I-50% Met and II-50% Met; and I-100% Met and II-100% Met [7].

Lipids were separated by TLC. Aliquots of 5 μl, each containing 50 mg lipid per 1 ml) were applied to Silica gel 60 plates (Merck). The solvent system used was chloroform–methanol–H_2O MiLi Q (65 : 25 : 4, v/v/v and 70 : 35 : 7, v/v/v). The chromatograms were treated using the following reagents: (1) 0.5% solution of vanillin (Sigma) in ethanol supplemented with 3% H_2SO_4 or iodine vapors [3]; (2) 0.5% solution of orcinol

(Sigma) in ethanol supplemented with 3% H_2SO_4 [3]; (3) 0.5% solution of ninhydrin (Sigma) in water-saturated butanol [4]; and (4) molybdenum reagent [7, 8].

Used as phospholipid standards were (1) 50 mg/ml solutions of reagents from Sigma: DPG (cardiolipin), phosphatidylglycerol (PG), PI, PE (cephalin), PC (lecithin), lyso-PC, phosphatidic acid (PA), phosphatidylserine (PS), and lyso-PS and (2) an equimolar mixture of standard solutions of PE, PC, PI, and lyso-PC (Supelco).

The mobility (*Rf*) and homogeneity of phospholipid preparations were assessed by two-dimensional TLC using Silica gel 60 plates and two systems of solvents: chloroform–methanol–H_2O MiLi Q, 65 : 25 : 4, v/v/v (direction I), and *n*-butanol–acetic acid–H_2O MiLi Q, 60 : 20 : 20, v/v/v (direction II) [7]. The chromatograms were treated with molybdenum reagent.

RESULTS AND DISCUSSION

The use of phospholipids as chemotaxonomic markers is largely limited by cultivation-dependent changes in their composition. For this reason, we cultured bifidobacteria in synthetic Eagle's medium (supplemented with 0.5% lactose and 0.05% ascorbic acid), which ensured high levels of reproducibility of the data under strictly standard conditions of biomass growth. Extracted lipids accounted for ~2.1% of the wet weight of bifidobacteria. Analysis of the composition of lipid extracts of bifidobacteria demonstrated the presence of polar lipids (glycolipids and phospholipids) and nitrogen-containing lipids with a free amino group.

Comparative TLC analysis of lipid extracts from *B. adolescentis* 94 BIM, *B. bifidum* 791, *B. longum* B 379 M, and standard phospholipid specimens demonstrated that the samples under study contained phosphorus-containing components, including those similar to phosphoglycerides in their chromatographic mobility (R_f), i.e., DPG (R_f = 0.51) and PG (R_f = 0.45). The system of solvents used in these experiments was chloroform–methanol–H_2O MiLi Q, 70 : 35 : 7, v/v/v.

Preparative chromatography of lipid preparations extracted from the biomass of *B. adolescentis* 94 BIM on silica gel columns I and II made it possible to obtain fractions containing polar lipids. Comparative TLC analysis of methanol fractions demonstrated the presence in lipid fractions of the following phosphorus-containing components, which are similar to phospholipids in their chromatographic mobilities (R_f values): PG (fractions III-Met and IV-Met), PI (fractions II-Met–IV-Met and I-10% Met), PC (fraction I-10% Met), lyso-PC (fractions III-Met and IV-Met, IV-10% Met, III-15% Met, and I-30% Met; lipid extract), PA (fractions I-Met and II-Met), and lyso-PS (fractions IV-10% Met and I-30% Met; lipid extract). DPG and PE were found predominantly in lipid extracts of *B. adolescentis* 94 BIM (Table 1).

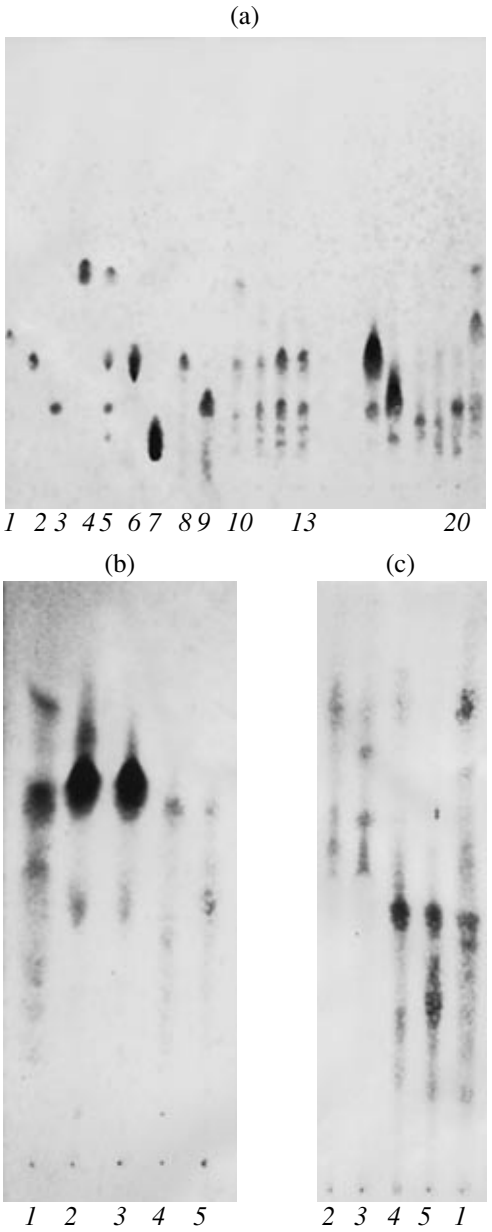

Fig. 1. Thin-layer chromatogram of 5-μl fractions (50 mg/ml) obtained by preparative column chromatography of the lipid extract of *B. adolescentis* 94 BIM (Column I) and the fraction I-Ac (Column II). (a) *1*, DPG; *2*, PG, *3*, PI; *4*, PE; *5*, mixture of PE, PC, PI, and lyso-PC; *6*, PC; *7*, lyso-PC; *8*, PA; *9*, lyso-PS; *10–13*, methanol fractions from Column I (I-Met, II-Met, III-Met, and IV-Met, respectively); *14–20*, methanol fractions from column II (I-5% Met, II-5% Met, I-10% Met, IV-10% Met, III-15% Met, III-20% Met, and I-30% Met, respectively). (b and c) *1*, lipid extract; *2*, IV-5% Met; *3*, I-10% Met; *4*, III-20% Met; and *5*, I-30% Met. Solvent system: chloroform–methanol–H_2O MiLi Q (65 : 25 : 4, v/v/v). Visualization: (a) and (b), treatment with molybdenum reagent; (c), treatment with orcinol.

Phospholipid to glycolipid ratios in fractions obtained by column chromatography were derived from analyses of thin-layer chromatograms, which were treated separately with the molybdenum reagent

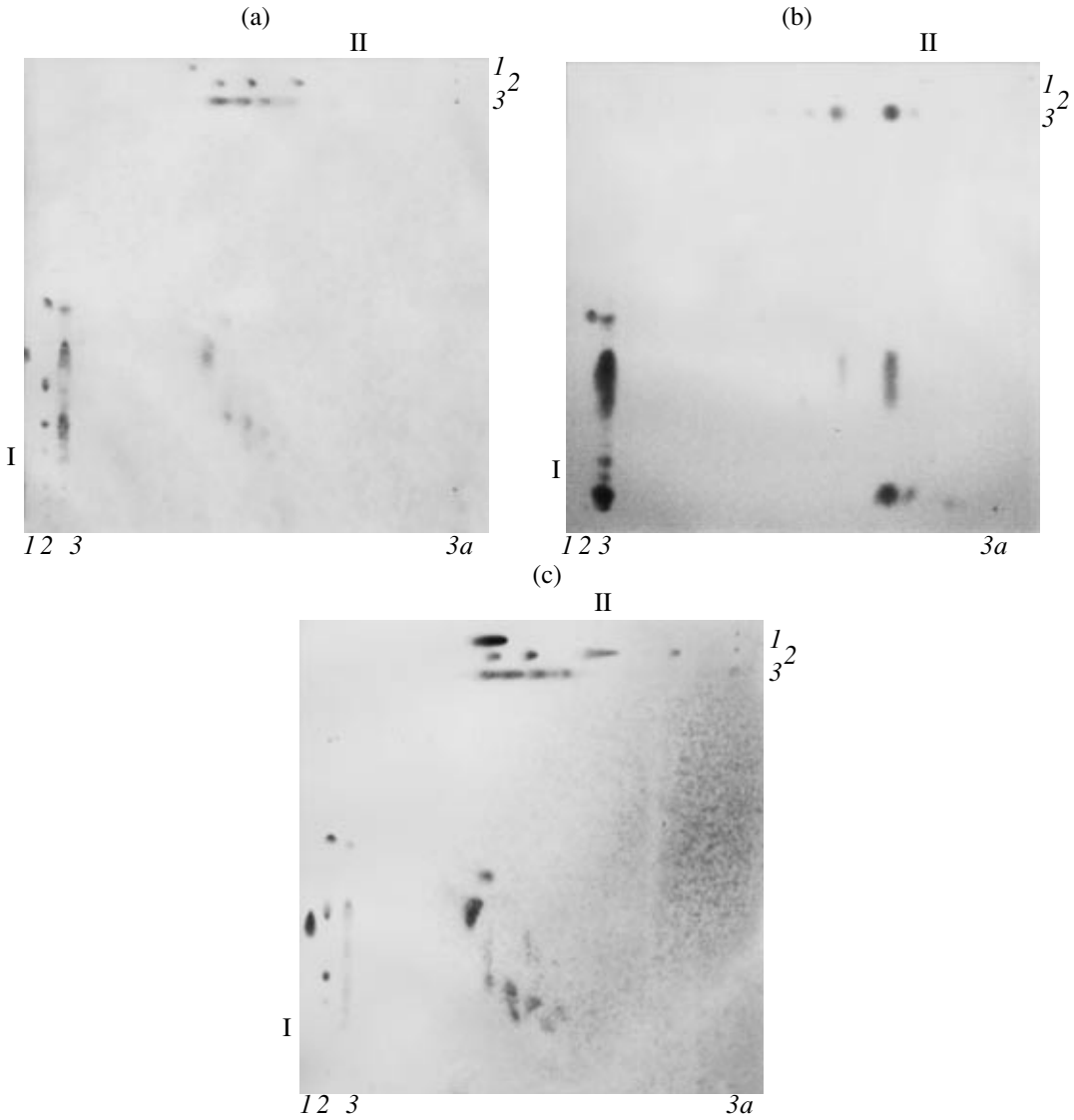

Fig. 2. Analysis of phospholipids (50 mg/ml) of *B. adolescentis* by two-dimensional thin-layer chromatography. Visualization: (a) and (c), treatment with molybdenum reagent; (b), treatment with ninhydrin. Designations: *1a* and *1b*, DPG (6 µl); 1*c*, PG (6 µl); *2*, mixture of PE, PC, PI, and lyso-PC (6 µl); *3*, (12 µl); *3a* (24 µl), lipid extract.

and orcinol (Fig. 1). Phospholipids dominated fractions IV-5% Met and I-10% Met, whereas fractions III-20% Met and I-30% Met primarily contained glycolipids.

Further analysis of polar lipids of *B. adolescentis* 94 BIM, *B. bifidum* 791, and *B. longum* B 379 M involved two-dimensional TLC. Phospholipid identification was performed based on qualitative reactions and comparison with standard markers. Our results demonstrated that DPG, PG, PE, and PC were dominant phospholipids of bifidobacteria, whereas PI and lyso-PC were present as minor components (Table 2, Fig. 2). The use of the solvent systems chloroform–methanol–H_2O MiLi Q, 65 : 25 : 4, v/v/v (direction I), and *n*-butanol–acetic acid–H_2O MiLi Q, 60 : 20 : 20, v/v/v (direction II), made it possible to separate and re-extract phospholipids present in the chromatograms in the form of compact spots.

Thus, preparative column chromatography and TLC allowed us to demonstrate that all representatives of the genus *Bifidobacterium* included in this study were characterized by the same composition of phospholipids, glycolipids, and unidentified lipids.

Judging by their significance in contemporary systematics, phospholipids are viewed as being equal to other taxonomically important components, such as sugars and amino acids of cell walls. In this connection, it is critical to identify chemotaxonomic markers among glyco- and phospholipids, which would be readily measured and reliable [9–11].

Data obtained by Exterkate and Veerkamp indicate that major phospholipids of *Bifidobacterium bifidum* var. *pennsylvanicus* are represented by DPG, PG, and polyglycerol phospholipids; mono-, di-, and triacyl-*bis*-(glycerophosphoryl)glycerols, alanylphosphatidylglycerol, and PA are found in considerably lower amounts. PG is a precursor of polyglycerol phosphate of lipoteichoic acid. Conversely, polar lipids of lactobacilli are largely represented by PG, whereas PA, DPG, lyso-PG, phosphoglycolipids, and diglycosyldiacylglycerol (DGDG) appear as minor components [5, 12, 13]. Mass-spectrometric studies of lipids from lactobacilli demonstrated that lipid extracts contained PG species—PG(37 : 2), PG(36 : 2), PG(35 : 1), PG(34 : 1), and PG(33 : 1)—but were devoid of nitrogen-containing phospholipids [5, 6, 14].

We were able to demonstrate that representatives of the genus *Bifidobacterium—B. longum* B 379 M, *B. bifidum* 791, *B. adolescentis* 94 BIM—are characterized by the presence of phospholipids that form a specific TLC profile. Studies of lipid preparations of bifidobacteria by two-dimensional TLC, involving qualitative parallel reactions with ninhydrin and orcinol (identifying, respectively, nitrogen-containing lipids with a free amino group and glycolipids), made it possible to determine both major phospholipids—DPG, PG, PE, and PC—and minor components (phosphorus-containing compounds, the content of which was significantly lower), i.e., PI and lyso-PC. It would seem that these compounds, together with glycolipids, may be viewed as genus-specific chemotaxonomic markers convenient for use in contemporary identification procedures developed for microorganisms of the genus *Bifidobacterium*.

Serodiagnostics and development of rapid tests for identifying causative agents of mixed infections (caused by representatives of normal microflora and opportunistic bacteria) constitute yet another field in which phospholipids and glycolipids may be used as chemotaxonomic markers. We believe that studies on the location of lipid and carbohydrate antigens in the cells of bifidobacteria are of considerable importance in this respect. Should the antigens be located at the surface of the bacterial cell, they would ensure close contact with immunocompetent cells (which is an indication of rapid recognition of antigens by defense system of the host) [15–17].

Further studies of the lipid components (including the chemical structure and biological role of the compounds) is expedient, because, in addition to their taxonomic importance, the lipids in question may be involved in specific cellular reactions that underlie the probiotic effects of bifidobacteria at the whole-body level [18–20]. Research into structure–activity relationships (i.e., the composition and function of phospholipids of bifidobacteria) constitutes another topical field of activity. Isolation from bifidobacteria of new biologically active substances (polysaccharides and enzymes, glycolipids and phospholipids, etc.) and their development, aided by high technologies, into efficient medicines for prevention and treatment of diseases constitutes a separate field of research activity.

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REFERENCES

1. Biavati, B., Sgorbati, B., and Scardovi., V., in *The Procaryotes*, Balows, A., *et al.*, Eds., New York: Springer, 1992, pp. 816–833.
2. Cummins, C.S., Cell Wall Composition in the Classification of Gram-Positive Anaerobes, *Int. J. Syst. Bacteriol.*, 1970, vol. 20, pp. 413–419.
3. Mordarska, H., Szponar, B., Gamian, A., and Pasciak, M., Taxonomic and Immunological Value of Lipid Markers of Actinomycetes and Actinomycetes-Like Bacteria, *Post. Hig. Med. Dosw*, 1995, vol. 49, pp. 161–170.
4. Mordarska, H., Zakrewska-Cherwinska, J., Pasciak, M., Szponar, B., and Rowinski, S., Rare, Suppurative Pulmonary Infection Caused by *Nocardiopsis dassonvillei* Recognized by Glycolipid Markers, *FEMS Immunol. Med. Microbiol*, 1998, vol. 21, pp. 47–55.
5. Drucker, D.B., Megson, G., Harty, D.W.S., Riba, I., and Gaskell, S.J., Phospholipids of *Lactobacillus* spp., *J. Bacteriol.*, 1995, vol. 177, pp. 6304–6308.
6. Exterkate, F.A., Otten, B.J., Wassenberg, H.W., and Veerkamp, J.H., Comparison of the Phospholipid Composition of *Bifidobacterium* and *Lactobacillus* Strains, *J. Bacteriol.*, 1971, vol. 106, pp. 824–829.
7. Novik, G.I., Astapovich, N.I., Grzhegorzhevich, A., and Gam'yan, A., Isolation and Comparative Analysis of Glycolipid Fractions in Bifidobacteria, *Mikrobiologiya*, 2005, vol. 74, no. 6, pp. 772–780.
8. Gamian, A., Mordarska, H., Ekiel, I., Ulrich, J., Szponar, B., and Defaye, J., Structural Studies of the Major Glycolipid from *Saccharopolyspora* Genus, *Carbohydr. Res.*, 1996, vol. 296, pp. 55–67.
9. Veerkamp, J.H. and Schaik, F.W., Biochemical Changes in *Bifidobacterium bifidum* var. *pennsylvanicus* After Cell Wall Inhibition. VII. Structure of the Phosphogalactolipids, *Biochim. Biophys. Acta*, 1974, vol. 348, pp. 370–387.
10. Schaik, F.W. and Veerkamp, J.H., Biochemical Changes in *Bifidobacterium bifidum* var. *pennsylvanicus* After Cell Wall Inhibition. VIII. Composition and Metabolism of Phospholipids at Different Stages and Conditions of Growth, *Biochim. Biophys. Acta*, 1975, vol. 388, pp. 213–225.
11. Veerkamp, J.H., Biochemical Changes in *Bifidobacterium bifidum* var. *pennsylvanicus* After Cell Wall Inhibition. IX. Metabolism and Release of Cellular Lipids in

the Presence of Antibiotics, *Biochim. Biophys. Acta*, 1976, vol. 450, pp. 277–287.

12. Schaik, F.W. and Veerkamp, J.H., Metabolic Relationships Between Phospho(Galacto)Lipids in *Bifidobacterium bifidum* var. *pennsylvanicus*, *FEBS Lett.*, 1976, vol. 67, pp. 13–16.
13. Veerkamp, J.H., Effects of Growth Conditions on the Lipid Composition of *Bifidobacterium bifidum* subsp. *pennsylvanicus*, *Antonie van Leeuwenhoek*, 1977, vol. 43, pp. 101–110.
14. Veerkamp, J.H., Biosynthesis of Phosphogalactolipids and Diphosphatidylglycerol in a Membrane Fraction of *Bifidobacterium bifidum* var. *pennsylvanicus, Biochim. Biophys. Acta*, 1976, vol. 441, pp. 403–411.
15. 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.
16. 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.
17. Kubler-Kielb, J., Novik, G., Adamik, B., Astapovich, N., and Gamian, A., Immuno-Chemical Study of Water-Soluble Polysaccharide Isolated from *Bifidobacterium adolescentis* 94-BIM, *FEBS'99,* Nice, June 19–24, 1999, Su/10.2/158.
18. Novik, G.I., Astapovich, N.I., Kyubler, I., and Gam'yan, A., Characterization of Polysaccharides Secreted by *Bifidobacterium adolescentis* 94 BIM, *Mikrobiologiya*, 2002, vol. 71, no. 2, pp. 205–210.
19. Vuyst, L.D. and Degeest, B., Heteropolysaccharides from Lactic Acid Bacteria, *FEMS Microbiol. Rev.*, 1999, vol. 23, pp. 153–177.
20. Mordarska, H. and Pasciak, M., A Simple Method for Differentiation of *Propionibacterium acnes* and *Propionibacterium propionicum*, *FEMS Microbiol. Lett.*, 1994, vol. 123, pp. 325–330.