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EXPERIMENTAL ARTICLES

Biological Activity of Polar Lipids from Bifidobacteria

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Abstract—Fractions of polar lipids have been isolated from bifidobacteria, and the immunoreactivity and serological specificity of glycolipids and phospholipids have been studied. Enzyme immunoassay (dot-EIA) of polar lipids demonstrates that the fractions of glycolipids and phospholipids of bifidobacteria are highly immunoreactive. Pronounced reactions of major glycolipids and phospholipids with a homologous polyvalent antiserum against *B. adolescentis* 94-BIM have been observed at antigen concentrations of approximately 100 ng. The antiserum contained high titers of specific antibodies against glycolipids and phospholipids of bifidobacteria, as demonstrated by heterogeneous solid-phase enzyme immunoassay (ELISA). Experimental data confirming the presence of subpopulations of specific antibodies (antiglycolipid and antiphospholipid) in the blood sera of immunized animals lead to the conclusion that the major glycolipids and phospholipids of bifidobacteria are specific markers appropriate for serological diagnostics.

Key words: bifidobacteria, glycolipids, phospholipids, biological activity.

Microorganisms of the genus *Bifidobacterium* are symbionts of the gastrointenstinal tract of humans and animals. According to current taxonomic understanding, the genus *Bifidobacterium* is classified with the family Actinomycetaceae of the order Actinomycetales. Bifidobacteria have provided a base for developing (1) a generation of medicines for the prevention and treatment of diseases, (2) pharmacologically active (functional) foodstuffs, and (3) food additives. Bifidobacteria exert antineoplastic, detoxifying, antipyrogenic, and immunomodulating effects. These effects are due to the bacteria's ability to (1) correct cell numbers in lymphocyte subpopulations of peripheral blood, (2) stimulate the phagocytic activity of neutrophils, (3) activate the generation of interferon, and (4) regulate lymphokine production [1–3].

The probiotic effects exerted by microorganisms of the genus *Bifidobacterium* are believed to be largely due to the organisms' biologically active glycoconjugates—the constitutive cellular components represented by polysaccharides, glycoproteins, glycolipids and phosphoglycolipids, and complexes of lipoteichoic acids with proteins. Glycoconjugates exert antineoplastic and immunomodulating effects (the administration of bifidobacteria triggers a cascade of immunological reactions) [4–6]. Serological studies indicate that the glycoconjugates of bifidobacteria are heterogeneous and may have diagnostic implications. At the moment, researchers are focused on analyzing the chemical structure of glycoconjugates, which are viewed as chemotaxonomic markers for the identification of microorganisms belonging to diverse systematic groups (including bifidobacteria). The heterogeneity of the chemical structure of glycoconjugates accounts for the character of their interactions with antibodies of polyclonal blood sera [7]. In addition to serving as antigenic determinants of bifidobacteria, glycoconjugates perform protective functions, defending cells from phagocytosis and the effects of antimicrobials. Data from the literature [8] indicate that glycolipids of the type *B. bifidum* var. *pennsylvanicus* are represented by galactolipids—mono-, di-, and trigalactosyl diglycerides; mono- and diacyl derivatives of monogalactosyl diglycerides; and monoacyl derivatives of digalactosyl diglycerides (monogalactosyl and digalactosyl monoglycerides are present in minor amounts). Diphosphatidylglycerol, phosphatidylglycerol, and polyglycerol phospholipids are the major phospholipids of *B. bifidum* var. *pennsylvanicus*. Mono-, di-, and triacyl-*bis*-(glycerophosphoryl)glycerol; alanylphosphatidylglycerol; and phosphatidic acid are present in considerably lower amounts [9, 10]. Studies of the immunoreactivity of polar lipids (glycolipids and phospholipids), which act as antigens involved in the specific mechanisms underlying the probiotic effects of bifidobacteria, are of unquestioned theoretical importance.

In this work, we sought to isolate polar lipids (glycolipids and phospholipids) from bifidobacteria and study their immunoreactivity.

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MATERIALS AND METHODS

Objects of the study. We used bifidobacterium strains *Bifidobacterium longum* B 379 M, *B. bifidum* 791 (courtesy of researchers of the Gabrichevskii 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).

Culturing. 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 CLY or TSB media. A physiologically active culture of bifidobacteria (generation III), grown on CLY or TSB media, served as inoculum. Bifidobacteria were grown under microaerophilic conditions at 37°C (thermostated chamber) for 24–48 h (steady-state growth phase). To check sterility, aliquots of the culture liquid were taken at intervals and seeded on blood agar and beef peptone agar; in addition, preparations of the bacteria were examined microscopically (according to standard techniques) in a Karl Zeiss microscope (Germany) at 1000×. Morphologically homogeneous cultures of rodlike bifidobacteria were used in the experiments. The cells were harvested by centrifugation (10000 *g*) of the culture liquid 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 PO_4^{3+}, 0.15 M NaCl, pH 7.3) at 8000 *g* (20 min), to eliminate components of the medium. A portion of the biomass was washed with H_2O MiLi Q and lyophilized. 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 [11, 12]. The investigation of the immunoreactivity of the polar lipids of bifidobacteria involved several stages.

Raising polyclonal antisera against *B. adolescentis* 94-BIM. Male rabbits aged 6 months, weighing 3.5–4 kg, were inoculated for 4 weeks with a suspension of lyophilized bifidobacteria (obtained by culturing on Eagle's and CLY media), with the inoculation dose being increased progressively from 0.1 to 6.4 mg/ml PBS. If an increase in blood antibody titers was required, the animals were given two additional administrations at the maximum inoculation dose. On the whole, the amount of bacteria introduced into the organism was approximately 20–25 mg/animal. The animals were killed via cardiac puncture. The blood was incubated at 37°C for 30 min, followed by additional incubation at 4°C for 4 h. It was subsequently centrifuged at 1000 *g* for 15 min. The serum was inactivated by heating at 56°C for 30 min. It was stored at –70°C.

Characterizing polar lipid antigenicity in immunologic tests: dot-EIA and ELISA. The immobilization of polar lipids on reaction carriers (nitrocellulose membranes and polystyrene plates) posed certain problems due to the lipids' exclusive solubility in organic solvents. The lipid antigen of bifidobacteria was dissolved in chloroform (semiquantitative dot-EIA) or methanol (ELISA).

In order to perform dot-EIAs, the lipid fraction (1000, 500, 100, 50, and 10 ng) was applied pointwise onto strips of nitrocellulose membrane (Micro Filtration System; pore diameter, 0.45 μm). Following the drying and blocking of the lipids with an 0.2% casein solution in 50 mM Tris–HCl (TBS; pH 7.4), the strips were washed three times with TBS and incubated (37°C, 12 h) with 100-fold dilutions of polyclonal rabbit antisera against *B. adolescentis* 94-BIM or blood sera of normal healthy animals (controls). Thereafter, the strips were washed three times in TBS and incubated (37°C, 2 h) with horseradish peroxidase-conjugated goat antirabbit immunoglobulin (DAKO), using a 2000-fold dilution. After the incubation was completed, the strips were washed three times in TBS and treated with a solution of the substrate, 4-chloro-1-naphthol (Sigma). The solution was prepared by dissolving 12 mg of the substrate in 4 ml methanol and adding 20 ml of TBS supplemented with 20 μl 30% H_2O_2. After 30–60 min of incubation with the substrate, the strips were read by visual examination; positive reactions appeared as light-blue to dark-blue spots, which corresponded to the sites of antigen application. The reaction was stopped by transferring the strips into PBS.

In order to perform ELISAs, aliquots (50 μl) of lipid samples (1 μg per aliquot) were introduced into the wells of polystyrene plates (Maxi Sorp, Nunc, or PV DF, Falcon), and the plates were left at 24°C for 4 h, allowing the methanol to evaporate completely. Thereafter, the antigens were blocked by a 2% solution of bovine serum albumin (BSA) in PBS. Polyclonal rabbit antisera against *B. adolescentis* 94-BIM or control samples were introduced into the wells (as 100-, 200-, 400-, 800-, 1600-, 3200-, and 6400-fold dilutions in PBS), and the plates were incubated at 4°C for 3 h. Thereafter, each well was washed three times with PBS, followed by the addition of horseradish peroxidase-conjugated goat antirabbit immunoglobulin (DAKO), using a 2000-fold dilution. The plates were incubated at 24°C for 1 h, after which each well was washed three times with PBS. The solution of the substrate (prepared by dissolving 3 mg *o*-phenylenediamine in 0.5 ml methanol and adding 9.5 ml citrate-phosphate buffer, pH 5.0) was then introduced into each well, and the plates were incubated for 20 min. The reaction was stopped by adding 2 M H_2SO_4. The results were analyzed on a Behring EL 311 microplate reader at 492 nm [7, 11]. Statistical processing of the data was carried out using conventional methods.

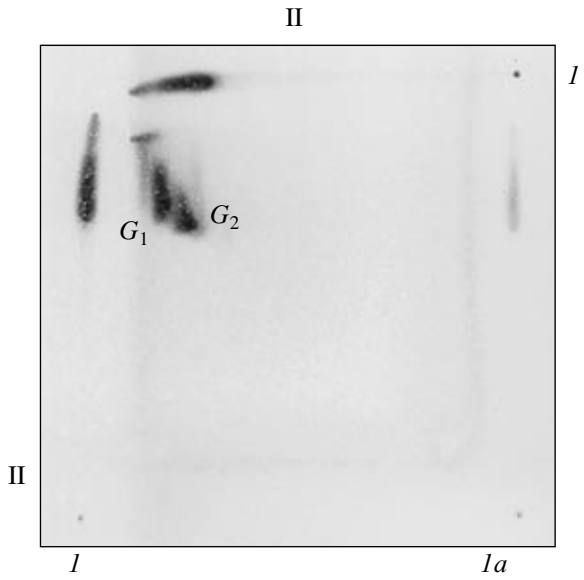

Fig. 1. Two-dimensional TLC of glycolipids of bifidobacteria (50 mg/ml; *1*, 12 µl; *1a*, 24 µl): *1* and *1a*, fraction II-5% Met (column II); G_1 and G_2, major glycolipids.

Solvent system: *n*-butanol–acetic acid–H_2O MiLi Q (60 : 20 : 20, v/v/v; directions I and II); detection: treatment with 0.5% solution of orcinol in ethanol supplemented with 3% H_2SO_4.

RESULTS AND DISCUSSION

The biomass of bifidobacteria (*B. longum* B 379 M, *B. bifidum* 791, and *B. adolescentis* 94 BIM) obtained as a result of 48-h submerged cultivation on synthetic Eagle's and TSB media, was used as material for lipid extraction. The efficiency of the extraction depended on the medium: it was approximately 2.06% for samples derived from cultures grown in the Eagle's medium and 4.04% for those grown in the TSB medium. Analysis of the composition of lipid extracts revealed the presence of neutral lipids (triacylglycerols, free fatty acids, and long-chain alcohols), polar lipids (glycolipids and phospholipids), and nitrogen-containing lipids (containing free amino groups). Preparative chromatography (column I packed with Silica gel 60, 70–230 mesh; Merck) of the extract of lipids of *B. adolescentis* 94 BIM (~586 mg; sequential elution with chloroform, acetone, and methanol) allowed the following fractions to be obtained: chloroform, I-Chl–IV-Chl (nonpolar lipids, long-chain alcohols and fatty acids); acetone, I-Ac–V-Ac (glycolipids); and methanol, I-Met–IV-Met (phospholipids). The fraction I-Ac (122.5 mg), containing glycolipids, was further separated by preparative column chromatography (column II; elution with a gradient of methanol in chloroform). As a result, we obtained preparations of the major glycolipids and phospholipids of *B. adolescentis* 94 BIM. Analysis of thin-layer chromatograms treated with orcinol and molybdenum reagent revealed fractions with a quantitative predominance of glycolipids and phospholipids. Highly purified glycolipids of *B. adolescentis* 94 BIM were largely present in fractions IV-Ac, V-Ac, II-5% Met, III-20% Met, and I-30% Met (Fig. 1). The yield of major glycolipids (fraction II-5% Met) relative to the total amount of glycolipids was approximately 16.33%. Highly purified phospholipids were predominant in fractions IV-5% Met and I-10% Met. The yield of phospholipids relative to the total amount of polar lipids was approximately 58%. Identification of polar lipids was performed by one-dimensional and two-dimensional TLC, employing phospholipid markers and qualitative reactions with ninhydrin (to identify nitrogen-containing lipids with free amino groups), and orcinol (to identify glycolipids). The predominant phospholipids in the cells of bifidobacteria were diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and phosphatidylcholine (PC). Minor phospholipids included phosphatidylinositol (PI) and lysophosphatidylcholine (lyso-PC).

The production of specific anti-lipid antibodies was studied using a polyvalent rabbit antiserum against *B. adolescentis* 94 BIM. A qualitative assessment of the immunoreactivity of the major glycolipids and phospholipids of bifidobacteria was performed using dot-EIA, a test intended for the rapid detection of antibodies in the bloodstream. Dot-EIA has certain advantages and disadvantages: although the test is semiquantitative and does not measure the level of antibodies, it is sufficiently sensitive to detect antibodies using microgram to nanogram amounts of antigens. We used lipid antigens in the range 10–1000 ng and 100-fold dilutions of test antisera in PBS. The results of the test demonstrated that glycolipid and phospholipid fractions of *B. adolescentis* 94 BIM are highly immunogenic. Pronounced reactions of major glycolipids (fractions II-5% Met and III-20% Met) and phospholipids (fraction I-10% Met) with the homologous polyvalent antiserum against *B. adolescentis* 94 BIM was observed at antigen concentrations of approximately 100 ng (Fig. 2). Studies of the level of specific antibodies against polar lipids of bifidobacteria in control sera (collected from the animals prior to immunization) gave negative or weakly positive results.

Quantitative determination of the level of specific antibodies in rabbit polyclonal antisera against *B. adolescentis* 94 BIM was performed by a heterogeneous solid-phase immunoassay (ELISA). The results confirmed that the antisera contained high levels of antibodies against glycolipids and phospholipids of *B. adolescentis* 94 BIM, which agrees with the data of dot-EIA.

Thus, we succeeded in isolating fractions of polar lipids of bifidobacteria and demonstrated that the major glycolipids and phospholipids were highly immunoreactive and exhibited serological specificity.

Constitutive nonspecific responses to infections, accounted for by the production of endogenous cytokines, are the primary (first line) defense mechanisms of an organism. Specific resistance (cellular and humoral)

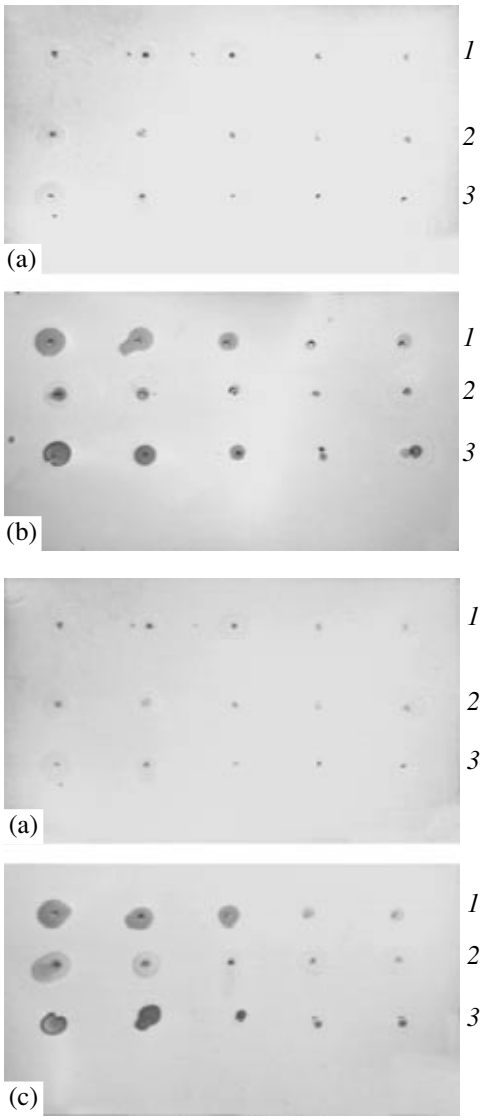

Fig. 2. Immunologic reactivity of major phospholipids and glycolipids with homologous polyvalent antisera against *B. adolescentis* 94 BIM (dot-EIA): *1*, fraction I-10% Met; *2*, II-5% Met; and *3*, III-20% Met.

(a) control blood serum (taken from the rabbit prior to immunization); (b) and (c) blood sera taken from rabbits immunized with cells of bifidobacteria grown on CLY medium (b) or Eagle's medium (c). The antigen was applied pointwise (left to right: 1000, 500, 50, and 10 ng).

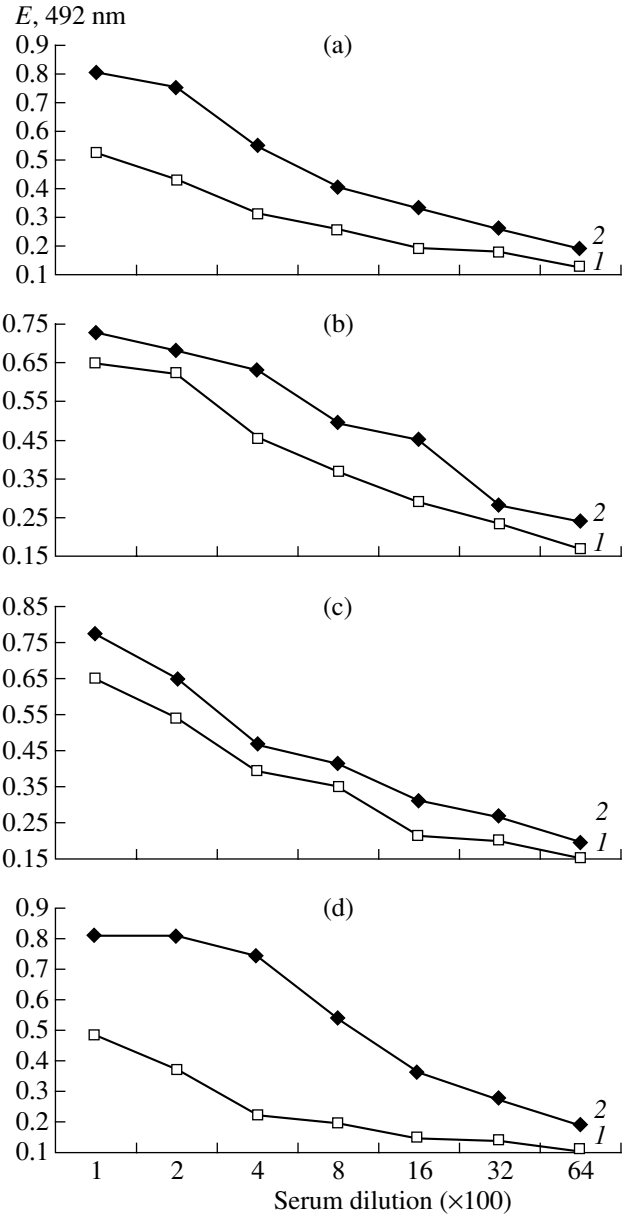

Fig. 3. Levels of antibodies recognizing major glycolipids and phospholipids in (*1*) homologous polyvalent antiserum against of *B. adolescentis* 94 BIM (taken from rabbits immunized with cells of bifidobacteria grown on Eagle's medium) and (*2*) control blood serum (taken from the rabbit prior to immunization), determined by ELISA: (a) fraction V-Ac; (b) fraction II-5% Met; (c) fraction I-10% Met; and (d) III-20% Met.

develops later on. In humans, constitutive resistance is characterized by the presence of interferons (IFN-α and IFN-γ) and tumor necrosis factor (TNF-α). TNF-α is the most important mediator of the immune response of a macroorganism; it activates macrophages, increasing their ability to produce nitrogen oxides. The dynamic state of the immunologic tolerance to antigens of symbiotic microflora, which is conserved over the course of evolution, plays a key role in the normal functioning of macroorganisms. Disturbances of immunologic tolerance (heralded by the appearance in the bloodstream of antibifidobacterial antibodies at high titers) result in prolonged destabilization of the self-regulating intestinal ecosystem. The average level of antibodies against antigens of bifidobacteria is considerably lower than the level of antibodies against other representatives of the intestinal microbiocenosis (enterococci, enterobacteria, etc.) [7, 12]. On the other hand, there is evidence that the introduction of bacterial cells into the host organism considerably stimulates interferon and TNF-α pro-

duction. For example, administration of bifidobacteria corrects the quantitative and functional parameters of B- and T-cellular immunity systems and induces the production of IFN-α and IFN-γ, ensuring the resistance of the organism to infections (which is supported by the indirect effects of immune mediators and the direct killing of viruses and bacteria). In our previous work [13], we demonstrated that physiologically active cells of *B. adolescentis* 94 BIM exert strong adaptogenic and immunity-correcting effects in irradiated organisms. The cytotoxic effects of bifidobacteria in a lymphocyte culture were not pronounced. They were, however, mitogenic *in vitro* and increased both the antimicrobial defense potential of phagocytes and the microbial capacity of blood. In addition, bifidobacteria contributed to the restoration of the synthesis of interleukin-1 (IL-1) and TNF-α, which are mediators of spontaneous and induced immune responses. Similar results were obtained with other representatives of actinomycetes. Administration of killed *Rhodococcus equi* cells into mice increased the production of cytokines (IL-4 and IFN-γ). It is also known that IFN-γ is involved in the formation and development of granulomas induced by trehalose trimycolate isolated from the biomass of *Tsukamurella paurometabolum* (*Gordona aurantiaca*) [7, 12–17].

There is evidence that the polar lipids of bacteria (e.g., major glycolipids of *Saccharopolyspora rectivirgula* and *R. equi*) can be used as interferon inducers. The production of TNF-α is induced by major and minor glycolipids of *R. equi*. Stimulation of interferon- and TNF-α-producing cells by immunomodulating substances caused a dose-dependent increase in cytokine production. The glycolipids of actinomycetes which are immunogenic and can be used in serodiagnostics have now been isolated and purified. These are: trehalose dimycolate, phenolic glycolipids, lipoarabinomannan, and polar lipooligosaccharides [7, 12, 14–17].

Enzyme immunoassay of polar lipids of *B. adolescentis* 94 BIM indicates that their glycolipid and phospholipid fractions are highly immunoreactive and exhibit serological specificity. Pronounced reactions of polar lipids with a homologous polyvalent antiserum against *B. adolescentis* 94-BIM were observed at antigen concentrations of approximately 100 ng. The experimental data indicate that the blood sera of immunized animals contain subpopulations of specific antibodies (anti-glycolipid and anti-phospholipid). Thus, the major polar lipids of *B. adolescentis* 94 BIM are specific markers showing promise as reagents for serodiagnostics. Determining the pool of antibodies specific to bacterial glycolipids and phospholipids by dot-EIA and ELISA may have a wide range of practical applications. These include rapid serological tests for the diagnosis of mixed infections caused by opportunistic microflora, and methods for identifying the causative agents of infection in patients at risk of developing a septic condition. In addition, when based on the use of polystyrene plates or nitrocellulose membranes, EIAs for determining the etiological factors of infection are faster (and undoubtedly more sensitive) than the traditional methods of microbiology. Immunologic tests complement the set of qualitatively novel approaches to diagnosing diseases caused by representatives of the order Actinomycetales. Other approaches include chemotaxonomic methods (based on identification of genus-specific markers, such as mycolic acids, major glycolipids and phospholipids), and methods of molecular systematics (involving the use of microbial probes and DNA hybridization) [7, 12, 14–18].

Studies of the involvement of lipids in the specific probiotic reactions of bifidobacteria imply that the chemical composition and structure of the polar lipids under study are correlated with their function. The isolation from bifidobacteria of new biologically active substances (polysaccharides, glycolipids, and phospholipids) and their development, aided by high technologies, into effective medicines for the prevention and treatment of diseases constitutes a separate field of research activity.

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