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EXPERIMENTAL ARTICLES

New Approaches for the Isolation of Bifidobacterial Strains, Their Molecular Characterization, and Assessment of Their Probiotic Potential

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Abstract—Six stably growing strains of bifidobacteria possessing probiotic properties were isolated from the feces of newborn children and animals. According to the results of molecular analysis, one strain was classified as *Bifidobacterium bifidum,* while five belonged to *Bifidobacterium animalis.* Initial identification of the strains was carried out using the primer pairs for the 16S rRNA gene (*g-Bifid-F/R, Bif164/662*) and for the *xfp* gene specific for bifidobacteria. Subsequent sequencing of complete genes encoding 16S rRNA synthesis in the isolates confirmed their species affiliation. The cultures exhibited high resistance to gastroenterological stress ($5 \le RD \le 10$) and may therefore be recommended as potential probiotics.

Keywords: isolation of bifidobacteria, molecular genetic identification, storage of bacteria, stress resistance

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Development of probiotic drugs containing a variety of probiotic microorganisms has recently become an area of considerable research interest [1]. Development of such drugs requires consideration, apart from the probiotic properties of bacterial strains, of their ability to interact with the host's own microflora [2]. An important component of probiotic drugs are bifidobacteria, which normally constitute a major fraction of the gastrointestinal tract microflora. A number of studies have characterized the principal taxonomic, morphological, and physiological properties of bifidobacteria [3, 4], as well as the qualitative and quantitative composition of bifidobacterial communities inhabiting the human intestine depending on the host's age, character of nutrition, and the climate zone [5–7].

Every species of bifidobacteria is known to be characterized with individual probiotic properties and has a narrowly specific effect on the human organism [8]. For instance, *B. breve* helps to decrease blood cholesterol levels [9], *B. infantis* suppresses the growth of pathogenic microorganisms [10], and *B. longum* augments the immune response [11]. The industry of probiotic drugs and functional nutrition has come to strongly rely on both the universal and the specific properties of bifidobacteria.

Isolation of novel strains of bifidobacteria is associated with considerable difficulties. The growth of bifidobacteria is often suppressed by lactic acid bacteria, which produce large amounts of organic acids and bacteriocin antibiotics [12]. Stable long-term maintenance of bifidobacteria in pure cultures can also be a problem [13].

The goal of the present work was to isolate novel strains of bifidobacteria from infants' and animals' feces, to identify them using molecular genetic techniques, and to analyze the probiotic potential of these cultures by determining their resistance to gastroenterological stress.

MATERIALS AND METHODS

Taking into account the physiological traits of bifidobacteria and their natural habitats, the study was performed with the specimens of feces of healthy newborn infants and various animals.

Composition and preparation of the media. Bifidobacteria were isolated using a liquid medium with the following composition (g/L): pancreatic hydrolysate of casein (Research Center of Applied Microbiology and Biotechnology, Russia), 30.0; yeast extract (Research Center of Applied Microbiology and Biotechnology), 5.0; glucose, 7.5; lactose, 2.5; cysteine, 0.5; NaCl, 2.5; $MgSO_4$, 0.5; ascorbic acid, 0.5; sodium acetate, 0.3; fish flour hydrolysate (Research Center of Applied Microbiology and Biotechnology), 50.0; Tween 80, 1.0 mL; 50% lactulose solution, 5.0 mL; hemin, 5 mg. Solid medium was prepared by adding 15–20 g/L agar.

Enrichment cultures of bifidobacteria were obtained by placing the feces (1 g) into a 10-mL vial containing 5 mL of sterile liquid medium. The vial was sealed with a sterile rubber stopper fastened with an aluminum cap. Anaerobic conditions were generated

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Table 1. Genus-specific oligonucleotide primers used for primary PCR identification of *Bifidobacterium* spp. strains

Gene	Name	Sequence (5'–3')	Primer length, bp	PCR product, bp	Reference
16S rRNA	*g-Bifid-F*	CTCCTGGAAACGGGTGG	17	556	Matsuki et al., 2003 [21]
	g-Bifid-R	GGTGTTCTTCCCGATATCTACA	21		
16S rRNA	*Bif164*	GGGTGGTAATGCCGGATG	18	523	
	Bif662	CCACCGTTACACCGGGAA	18		
xfp	Xfp-*F*	CGGCTGGCAGTCCAACAA	18	704	Piksasova, 2009 [22]
	Xfp-*R*	GGTTGTTCTTGATGATGTCGG	21		

by replacing air with sterile argon via a syringe. Bacteria were cultured at 37°C for 1 to 3 days until signs of growth were visible (e.g., cloudiness, change of color, gas formation).

Isolation of pure cultures. To isolate pure cultures, a 0.1 mL aliquot of an enrichment culture obtained as described above was plated on solid medium using the Koch's method to obtain individual colonies. Bacteria were grown at 37°C in anaerobic boxes using Genbox packages (BioMerieux, France). Further research was performed only with the colonies exhibiting the properties characteristic of bifidobacteria: gram-positive, oxidase- and catalase-negative rods of typical morphology. Material from individual colonies was transferred into 10-mL vials with liquid medium.

Storage of pure cultures. For long-term storage, cells were frozen at –70°C in skim milk supplemented with 5% glucose and 5% sucrose, and dried in a Free Zone lyophilizer (Labconco) at the temperature of –51°C and the pressure of 49 kPa for 24 h. Lyophilized cultures were stored in a refrigerator at 4°C.

Molecular identification of bifidobacteria. DNA was isolated from bacterial biomass as described in [14]. Prior to DNA isolation, cell biomass was concentrated by centrifugation in Eppendorf tubes (8000 *g*, 5 min), and the supernatant was discharged. Each specimen was washed twice with 1 mL of sterile physiological saline to remove the medium, which could potentially inhibit PCR.

Genus-specific polymerase chain reaction (PCR). Primary identification of the isolated pure cultures was performed by PCR with the primers specific to bifidobacteria (Table 1). The primers were synthesized by Syntol (Moscow, Russia). Amplification was performed in the reaction mixture (25 μL) containing the following: 1× PCR buffer (Fermentas, Lithuania) with KCl and $(NH_4)_2SO_4$ in amounts required for the efficient functioning of *Taq* polymerase, 3 mM $MgCl_2$, 400 mM each dNTP, 10 pmol each primer (three pairs), 10 μL DNA template, and 0.3 μL (0.3 U) *Taq* polymerase. The reaction was performed using a GeneAmp 9700 thermal cycler (Applied BioSystems, United States) according to the following protocol: initial DNA denaturation, 5 min at 95°C; 35 PCR cycles of 20 s at 95°C, 30 s at 57°C, and 40 s at 72°C; and final elongation for 4 min at 72°C. The annealing temperature was selected based on the properties of the individual primer pairs. After the efficiency of each primer pair was analyzed individually, it was determined that 57°C was an optimal annealing temperature for all three primer pairs. The sizes of the expected PCR products are given in Table 1.

After electrophoresis in a 1% agarose gel, PCR products were visualized by staining with the ethidium bromide intercalating dye (5 mg/mL). The standard 100 bp ladder (#SM0403, Fermentas) was used as a molecular size marker.

Species identification by the 16S rRNA gene sequencing. For the purposes of sequencing, DNA fragments representing complete 16S rRNA genes were amplified by PCR using a universal primer system: Univ11F (5'AGAGTTTGATCMTGGCTCAG) and Univ1492R (5'TACGGYTACCTTGTTACGACTT) [15]. The reaction mixture (50 μL) had the following composition: 1× buffer for Bio*Taq* DNA polymerase (17 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl pH 8.8, 2 mM $MgCl_2$), 12.5 nmol each dNTP, 50 ng DNA template, 5 pmol primers, and 3 units Bio*Taq* polymerase (Dialat Ltd., Russia). The PCR protocol was as follows: the initial cycle of 9 min at 94°C, 1 min at 55°C, and 2 min at 72°C; 30 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C; and final elongation for 7 min at 72°C. The products of PCR were analyzed by electrophoresis in a 0.8% agarose gel at 6 V/cm. The PCR products were isolated and purified from the gel using a Wizard PCR Preps kit as recommended by Promega (United States).

Sequences of the PCR products representing the 16S rRNA genes were determined using Sanger's approach [16] with a Big Dye Terminator v.3.1 kit on an automated ABI PRISM 3730 DNA analyzer (Applied Biosystems, United States) according to the manufacturer's recommendations. The fragments were sequenced in both directions using universal primers [15]. The primary comparison of the 16S rRNA gene sequences of the isolated strains to homologous sequences available from the GenBank database was performed using the BLAST software package [17].

Isolated and identified cultures were submitted into the Collection of Cultures of the Department of Microbiology, Moscow State University (MD MSU) and assigned their respective accession numbers.

Determining the resistance of bifidobacteria to the conditions of the gastrointestinal tract. The effects of the gastric fluid and bile acids on the isolated bacteria under conditions of the gastrointestinal tract were studied in vitro as proposed in [18]. Bacterial strains were cultured in liquid medium under anaerobic conditions for 24–48 h (stationary phase) at 37°C and pH 6.5

To analyze resistance to gastric acids, 100 μL of a stationary-phase culture was added to 1 mL artificial gastric fluid (1 : 11 dilution), and the mixture was incubated in an anaerobic jar at 37°C for 10, 30, or 60 min. In the control variants, a culture aliquot was mixed with 1 mL medium. Following incubation, sequential tenfold dilutions (from 10^{-2} to 10^{-10}) in fresh sterile medium were prepared with both the control and test cultures, plated on solid medium, and incubated in an anaerobic jar for 24–48 h at 37°C to determine the number of surviving cells (by CFU) in either variant. The degree of resistance to gastric fluid (RD) was calculated using the formula $RD = n1/n2$, where $n1$ and $n2$ were the CFU numbers per 1 mL of the control and test specimens, respectively. The resistance degree was graded as proposed in [18]: $RD \leq 5$, very good; $5 < RD \leq 10$, good; $10 < RD \leq 15$, acceptable; $RD > 15$, unacceptable.

The artificial gastric fluid had the following composition (g/L): NaCl (Sigma S9625), 2.2; L-lactate (Sigma L1750), 9.9 (0.11 M); porcine pepsin (Sigma P7125), 3.5 (600–1800 U/mg); pH 2.7 ± 0.02 (adjusted with 35% NaOH); pH after 1 : 11 dilution, 3.10 ± 0.10 (pH was verified for each culture).

To analyze the resistance to bile acids, a 100-μL aliquot of a stationary-phase culture was added to 1 mL artificial intestinal fluid (1 : 11 dilution). In the control variants, a culture aliquot was mixed with 1 mL medium. The tests were performed for 5 h similarly to those evaluating the effects of the gastric fluid.

The artificial intestinal fluid had the following composition (g/L): bile salts (porcine bile, Sigma B8631), 3.3 (final concentration, 0.3%); $NaHCO_3$ carbonate buffer (Sigma S8875), 16.5 (final concentration, 1.5%); pH 6.3.

The resistance degree was determined using the same method as for the gastric fluid, as proposed in [18].

Statistical analysis. The experiments were performed in three independent replicates, each of them including three parallel tests. Data were analyzed using the conventional Student's *t*-test with the probability threshold of $P > 0.05$.

Reagents used for bacterial cultures and test assays were at least of the analytically pure grade.

RESULTS AND DISCUSSION

Isolation and identification of bacteria of the genus *Bifidobacterium*. For the purposes of *Bifidobacterium* isolation, 90 specimens of feces were collected from newborn infants and animals. Each specimen was placed in an individual vial, frozen, and stored at −20°C. Most enrichment cultures grew well on the special medium for bifidobacteria. However, after pure cultures were isolated from individual colonies obtained on petri dishes under anoxic conditions, their growth often stopped after two or three passages. Such cultures were rejected, and further analysis involved only the stably growing strains whose morphology conformed to the description of bifidobacteria.

From the collected specimens, 43 stably growing pure cultures were initially isolated. Based on the results of PCR with genus-specific primers (Table 1), only eight of them were tentatively identified as representatives of the genus *Bifidobacterium*. Three of these strains were isolated from infants' feces, and five strains were isolated from animals' feces. Initial description of cell morphology for these strains was done using the Bergey's Manual [19]. The results of morphological characterization are summarized in Table 2. Decisive identification of the genus and species affiliation of the isolated cultures was performed based on the complete sequences of their 16S rRNA genes. The nearly complete sequences obtained for the 16S rRNA genes amplified from the isolated strains were analyzed by comparison to homologous sequences available from the GenBank database. The results of a BLAST search performed with the determined sequences (Table 3) showed that only six of the eight isolated strains belonged to the genus *Bifidobacterium*, while the other two belonged to *Lactobacillus*. Molecular genetic analysis identified one of the *Bifidobacterium* strains as *B. bifidum* and five strains as *B. animalis*. Both *Lactobaccilus* strains belonged to the species *L. plantarum*.

Thus, we found the use of genus-specific PCR with the relevant specific primers described in the literature (Table 1) to be insufficient for reliable genus identification in bifidobacteria. In particular, in our experiments, 8 of 43 isolated pure cultures were identified as *Bifidobacterium* strains by genus-specific PCR; however, analysis of their 16S rRNA gene sequences showed that only six strains actually belonged to this genus, while two other strains were found to belong to the genus *Lactobacillus*. Therefore, the conventional genus-specific primers are in fact insufficiently specific and may produce false positive results. Ultimate identification of isolated bifidobacterial strains should rely on sequencing of complete 16S rRNA genes.

Assessment of bifidobacterial resistance to gastric fluid in vitro. All isolated strains of bifidobacteria were tested for resistance to artificial gastric fluid (Table 4). All strains showed high level of resistance ($5 < RD \leq 10$) during the first 30 min of incubation with gastric fluid, and the strain *B. bifidum* MD MSU no. 468 showed a good survival rate ($RD = 10$), even after a 60-min exposure (Table 4).

Assessment of bifidobacterial resistance to intestinal fluid in vitro. All six newly isolated strains of bifidobacteria, which have shown high survival rates in the

Table 2. Characterization of bifidobacteria isolated from human and animal feces

Source	Colony appearance	Cell morphology	Identification by the 16S rRNA gene sequence
Infant feces	Round milk-white colonies, elevated, medium-sized	Long slender rods with swollen ends, slightly curved	*B. bifidum* MD MSU no. 468
Infant feces	Small, round, beige	Small polymorphic swollen rods	*B. animalis* MD MSU no. 469
Infant feces	Small, elevated, beige	Small rods slightly swollen at the ends	*B. animalis* MD MSU no. 470
Goat feces	Large round colonies, cream-colored	Long rods, slightly swollen at the ends	*B. animalis* MD MSU no. 471
Rabbit feces	Convex colonies with a smooth surface	Slender V-shaped rods	*B. animalis* MD MSU no. 472
Goat feces	Large, glistening, creamy milk-colored colonies	Large rods with swollen ends, occasionally branched	*B. animalis* MD MSU no. 473
Goat feces	Glistening convex colonies with a smooth surface	Slender rods with claviform ends	*L. plantarum* MD MSU no. 501
Piglet feces	Small semitransparent colonies	Small polymorphic rods	*L. plantarum* MD MSU no. 502

presence of gastric fluids, were tested for viability following exposure to artificial intestinal fluid. In agreement with the purposes of this study—a search for bifidobacterial strains that could be used as components of fermented dairy products with a high probiotic potential—such strains should be able to pass through the stomach and the intestine without a considerable decrease in cell numbers. After passing the stomach, microbial cells traveling along the gastrointestinal tract are exposed to the contents of the intestine; in our study, this was simulated by exposure to artificial intestinal fluid. For all isolated cultures, the resistance values lied in the range of $5 < RD \leq 10$, indicating their good and very good survival in the intestine (Table 4).

Our study confirmed that the share of bifidobacterial stains that can be stably maintained as pure cultures rarely constitutes more than 10% of all strains with characteristic morphology isolated from natural specimens obtained from human or animal intestine. Using natural specimens, we obtained 90 enrichment cultures and were able to isolate from them only six strains of bifidobacteria stably growing as pure cultures. Apparently, in such natural ecological niches as human and animal feces, bifidobacteria form communities with uncertainly defined trophic relationships and receive some components required for their growth from companion microorganisms, which is why only a small portion of these bacteria can be stably maintained under laboratory conditions as pure cultures. This notion is also confirmed by the data on the biodiversity of human intestinal microflora comprising, by different estimates, 1200 to 1400 species. However, less than a half of them can be isolated as pure cultures [20]. Therefore, development of appropriate media and culture conditions for numerous currently unculturable species is an important direction of further research.

For the practical purposes of medicine and food industries worldwide, there is a demand for novel strains of bifidobacteria that could be considered as probiotic. In this work, we have isolated six novel strains resistant to gastric and intestinal fluids, making it possible to consider them as potential candidates for the development of probiotic cultures.

It should be stressed that some of the isolated strains identified as bifidobacteria based on morphological, microbiological, and conventional molecular genetic data did not actually belong to this genus. In particular, among the eight strains that had been initially identified as bifidobacteria based on their characteristic morphology and positive PCR with genus-specific primers (Table 1), two strains were found to belong to the genus *Lactobacillus* after complete 16S rRNA gene sequences were determined (Table 3), which further confirms that final identification of newly isolated bifidobacterial strains should rely on sequencing of the marker genes.

In conclusion, we should point out that isolation of new strains of bifidobacteria from natural sources remains a challenging task. Among the initially obtained 90 pure cultures with characteristic morphology, only 46 strains grew stably after sequential transfers. Further screening with genus-specific primers described in previous publications [21, 22] reduced the number of potential *Bifidobacterium* strains to

Table 3. Results of BLAST analysis of the isolated strains of bifidobacteria and lactobaccilli and their GenBank accession numbers

Specimen	Primers	Number of nucleotides determined	Results of BLAST search	GenBank acc. no.
B. bifidum MD MSU no. 468	357f-1492r	1135	99% *B. bifidum* IDCC 4201	KJ160509
B. animalis MD MSU no. 469	27f-519r; 357f-1492r; 27f-1100r	1465	100% *B. animalis* subsp. *lactis* ATCC 27673	KJ160510
B. animalis MD MSU no. 470	27f-519r; 357f-1492r; 27f-1100r	1465	100% *B. animalis* subsp. *lactis* ATCC 27673	KJ160511
B. animalis MD MSU no. 471	27f-519r; 357f-1492r; 27f-1100r	1465	100% *B. animalis* subsp. *lactis* ATCC 27673	KJ160514
B. animalis MD MSU no. 472	27f-519r and 357f-1492r; 27f-1100r	1465	100% *B. animalis* subsp. *lactis* ATCC 27673	KJ160515
B. animalis MD MSU no. 473	357f-1492r	1135	100% *B. animalis* subsp. *lactis* ATCC 27673	KJ160516
L. plantarum MD MSU no. 501	357f-1492r; 27f-1100r	1465	99% *L. plantarum* 16, complete genome	KJ160512
L. plantarum MD MSU no. 502	357f-1492r; 27f-1100r	1465	99% *L. plantarum* 16, complete genome	KJ160513

Table 4. Resistance of the isolated strains of bifidobacteria to gastric and intestinal fluids

Species name	Resistance to gastric stress			Resistance to intestinal stress
	10 min	30 min	60 min	5 h
B. bifidum MD MSU no. 468	RD = 1.0 (very good)	RD = 1.0 (very good)	RD = 10.0 (good)	RD = 1.0 (very good)
B. animalis MD MSU no. 469	RD = 1.0 (very good)	RD = 8.0 (good)	RD = 10.0 (good)	RD = 4.0 (very good)
B. animalis MD MSU no. 470	RD = 1.3 (very good)	RD = 7.0 (good)	RD = 11.0 (good)	RD = 8.0 (good)
B. animalis MD MSU no. 471	RD = 1.0 (very good)	RD = 1.5 (very good)	RD = 3.1 (very good)	RD = 4.0 (very good)
B. animalis MD MSU no. 472	RD = 1.3 (very good)	RD = 8.0 (good)	RD = 38.0 (unacceptable)	RD = 6.0 (good)
B. animalis MD MSU no. 473	RD = 1.0 (very good)	RD = 1.5 (very good)	RD = 10.0 (good)	RD = 4.0 (very good)

RD, resistance degree [19].

eight, and two of those were subsequently rejected as lactobaccilli based on the complete sequence of their 16S rRNA genes. Thus, among 90 isolates obtained as pure cultures, only six strains were identified as bifidobacteria with the required probiotic properties.

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