RESULTS OF THE STUDY OF BIOLOGICAL AND MOLECULAR GENETIC PROPERTIES OF BIFIDOBACTERIUM STRAINS – PROBIOTIC PRODUCERS

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Abstract. For a long time, the strains *Bifidobacterium bifidum* 1, *Bifidobacterium bifidum* 791, and *Bifidobacterium longum* 379 have been successfully used as probiotic producers; the conclusions about the possibility of their use, their role and functions in the human body were made empirically based on indirect signs. At present, high-tech equipment and modern research methods allow to conduct a complete study of the properties and characterization of strains; it is regulated by modern regulatory documents. In our work we applied the classical bacteriological method, MALDI TOF mass spectrometry method, NGS whole genome sequencing. The analysis of 27 clones of the strains revealed that they have typical morphological properties and stable biochemical profiles; the lists of ionized protein masses (so-called «strain markers») were determined. The whole genomes of strains deposited by us in GenBank in 2015–2017 were analyzed for the first time; we found that they do not contain pathogenicity genes, integrated plasmids, determinants of transmissible antibiotic resistance, and belong to phylogenetic clusters formed by probiotic-producing strains. It was shown that *B. bifidum* 1 and *B. bifidum* 791 have a pronounced ability to consume glycans of intestinal mucus, while *B. longum* 379 can efficiently consume plant glycans. The genomes of *B. bifidum* 791 and *B. longum* 379 strains contain genes for the synthesis of the most important neurometabolites of tryptophan and folic acid; the genome of *B. bifidum* 791 strain contains genes for the synthesis of lasso peptide and flavucin, class I lantibiotics with a wide spectrum of antimicrobial and antiviral activity.

Keywords: *Bifidobacterium*, producer strains, probiotics, whole genome sequencing, MALDI TOF mass spectrometry, bacteriocins.

List of Abbreviations

BLAST – basic local alignment search tool (a family of computer programs used to search for homologues of proteins or nucleic acids)
CDS – coding sequence
EC – enzyme classification
GH – glycosil hydrolase
MALDI – matrix-assisted laser desorption/ionization
MSP – main spectra
RAST – rapid annotation using subsystems technology
SNP – single nucleotide polymorphism
TOF – time of flight
α-CHCA – α-Cyano-4-hydroxycinnamic acid
PM – pathogenic microorganisms
OM – opportunistic pathogens
SCFAs – short-chain fatty acids

Introduction

For a long time, strains *Bifidobacterium bifidum* 1, *Bifidobacterium bifidum* 791, and *Bifidobacterium longum* 379 have been successfully used as probiotic producers (Belova *et al.*, 2017; Tochilina *et al.*, 2016; Belova *et al.*, 2016; Soloveva *et al.*, 2012). The conclusions on the possibility of their use as well as their role and functions in the human body were made empirically based on indirect signs. At present, the availability of high-tech equipment and modern research methods, such as MALDI TOF mass spectrometry and whole genome sequencing, allows to conduct a complete study

of the properties and characterization of strains. In this regard, according to modern regulatory documents, the characteristics of producer strains should be supplemented with data on the stability of their properties, the absence of pathogenicity genes, integrated plasmids, transmissible antibiotic resistance genes, genetically determined metabolic potential, and the ability to synthesize biologically active substances (Guidelines for the control of biological and microbiological factors, 2011; Guidelines for the sanitary and epidemiological assessment of the safety and functional potential of probiotic microorganisms, 2010).

The knowledge about the characteristics of the metabolism of probiotic-producing strains, the spectrum of their enzymes and preferred sources of nutrition within the framework of evidence-based medicine contribute to the development of a personalized approach to prescribing probiotic therapy, taking into account the properties of each strain included in the drug or product, in a particular pathology accompanied by certain metabolic disorders associated, among other things, with changes in the structure and functions of the patient's microbiome. The presence of a wide range of bacterial hydrolases contributes to the most efficient absorption of food by the macroorganism, including complex carbohydrates of plant origin with the formation of a range of valuable metabolites such as short-chain fatty acids (SCFAs), which have a complex positive effect on human health (Kornienko, 2016; Oleskin & Shenderov, 2016). The ability of producer strains to synthesize neurotransmitters and their precursors affects the concentration of the most important neurotransmitters in the human body, the lack of which causes brain disorders and mental changes (Oleskin & Shenderov, 2019). A strain-specific property is the synthesis of bacteriocins – antimicrobial peptides that cause a pronounced antibacterial and antiviral activity of microorganisms, which allows probiotic strains to take part in the formation of «joint immunity», that is, to inhibit and eliminate opportunistic and pathogenic flora together with macroorganism's immunity factors (Oleskin & Shenderov, 2019; Arena *et al.*, 2018; Kim *et al.*, 2018; Lei *et al.*, 2016; Majamaa *et al.*, 1995; Soloveva *et al.*, 2021).

In this regard, there is no doubt about the relevance of the in-depth studies of the biological and molecular genetic properties of probiotic strains of bifidobacteria used for the production of medicines and foodstuffs in terms of assessing their probiotic and metabolic potential, the ability to synthesize glycosyl hydrolases, neurotransmitters, and bacteriocins.

The purpose of the study is an in-depth study of the biological properties and analysis of the genome features of producing probiotic strains *Bifidobacterium bifidum* 1, *Bifidobacterium bifidum* 791, *Bifidobacterium longum* 379.

To achieve this goal, it was necessary to solve the following tasks: to conduct profiling of bacterial proteins of strains using the Bruker MALDI TOF mass spectrometer and Biotyper software to identify individual characteristics; to evaluate the stability of the biochemical properties of strains; to analyze whole genome sequences of strains for pathogenicity determinants, antibiotic resistance genes, and integrated plasmids; evaluate the genetically determined metabolic potential of strains, their ability to synthesize glycosyl hydrolases, neurotransmitters, and bacteriocins; to determine the phylogenetic relationships of the studied strains with other strains of these species deposited in GenBank, including probiotic-producing strains; to determine characteristics of pan- and core genomes of all strains of *B. bifidum* and *B. longum* deposited in GenBank; to conduct a comparative analysis of the similarity of the amino acid sequences of proteins determined by the core genome genes of the studied strains and other strains of these species deposited in GenBank, including probiotic-producing strains.

Materials and Methods

The study was approved by the Ethics Committee of Privolzhsky Research Medical University.

Strains used in the study

The strains *B. bifidum* 1 (10 clones = ampoules), *B. bifidum* 791 (10 clones = ampoules),

B. longum 379 (10 clones = ampoules) were used in the study.

B. bifidum 1 is a production strain used in the production of bifid-containing probiotics registered in the Russian Federation (GPM. 1.7.1.0003.15) (General Pharmacopoeia Monograph, 2015), deposited in the State collection of pathogenic microorganisms (collection number of the strain – No. 900791) and the State collection of normal microflora microorganisms of G.N. Gabrichevsky Moscow Scientific Research Institute of Epidemiology and Microbiology (Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing). Academician I.N. Blokhina Nizhny Novgorod Scientific Research Institute of Epidemiology and Microbiology (formerly – Gorky Scientific Research Institute of Epidemiology and Microbiology) received the strain in the 1970s during the joint work with G.N. Gabrichevsky Moscow Scientific Research Institute of Epidemiology and Microbiology on the production of Bifidumbacterin.

B. bifidum 791 is a production strain used in the production of bifid-containing probiotics registered in the Russian Federation (GPM.1.7.1.0003.15) (General Pharmacopoeia Monograph, 2015), deposited in the Russian national collection of industrial microorganisms (VKPM) (deposit number – No. B-3300) and the State collection of normal microflora microorganisms of G.N. Gabrichevsky Moscow Scientific Research Institute of Epidemiology and Microbiology. The strain was transferred to Academician I.N. Blokhina Nizhny Novgorod Scientific Research Institute of Epidemiology and Microbiology (formerly – Gorky Scientific Research Institute of Epidemiology and Microbiology) together with the *B. bifidum* 1 strain for the production of fermented milk Bifilact.

B. longum 379 is a production strain used in the production of bifid-containing probiotics registered in the Russian Federation (GPM. 1.7.1.0003.15) (General Pharmacopoeia Monograph, 2015), the Russian national collection of industrial microorganisms (VKPM) (deposit number – No. B-2000) and the State collection of normal microflora microorganisms of G.N. Gabrichevsky Moscow Scientific Research Institute of Epidemiology and Microbiology. It was transferred to Academician I.N. Blokhina Nizhny Novgorod Scientific Research Institute of Epidemiology and Microbiology (formerly – Gorky Scientific Research Institute of Epidemiology and Microbiology) in 1989 during the joint work.

Cultivation of strains

To restore and sieve strains of *Bifidobacterium,* after opening the ampoule, the lyophilic mass was poured with 2 ml of bifidum medium (nutrient medium for the isolation and cultivation of bifidobacteria, dry, Obolensk), transferred to a sterile test tube with 9 ml of bifidum medium and incubated at 38 ± 1°C for 72 hours (the first generation of the strain). After 2 days, 2 ml of the first generation were inoculated into 50 ml of bifidum medium and incubated at 38 ± 1°C for 72 hours (the second generation of the strain).

Next, 1 ml of the second generation of the strain was titered in bifidum medium poured in a high column into 9 ml test tubes to a dilution of 10^{-11}. Dilutions of 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} were used to conduct 1 ml inoculations onto a plate medium for cultivating bifidobacteria (nutrient medium for determining and counting bifidobacteria, dry, State Research Center for Applied Microbiology and Biotechnology, Obolensk) and incubated for 72 hours at 38 ± 1°C under anaerobic conditions using GasPak Anaerobe Gas Generating Pouch System with Indicator, USA.

The morphology of grown colonies of microorganisms was evaluated; 10–12 colonies of each strain were selected for the next stage of the study: a sample from each colony was applied to 3 target cells for subsequent mass spectrometry, and the remainder was seeded in a bifidum medium for subsequent biochemical identification. Microscopy of Gram-stained smears was performed using a Micros MC500 laboratory microscope (Micros, Austria). Cultures that had Score values of 2.100 or more according to the results of mass spectrometry were selected for biochemical identification.

Strain identification and bacterial protein profiling

Identification and profiling of bacterial proteins of strains weree carried out using an Autoflex speed LRF time-of-flight MALDI mass spectrometer (Bruker Daltonics, Germany) equipped with a modified solid-state laser. All measurements were performed in a linear mode, detecting positive ions. To accumulate mass spectra, the laser radiation power was set at the level of the minimum threshold value sufficient for the desorption-ionization of the sample. The mass spectrometer parameters were optimized for the m/z range from 2000 to 20000. To obtain mass spectra suitable for identification, the following parameters were set in the device settings: summation of 10 series of spectra with 50 laser pulses each. External calibration was performed using a bacterial test standard (Bruker Daltonics, Germany) with α-cyano-4-hydroxycinnamic acid (α-CHCA) as a matrix. Sample preparation of cultures of the studied microorganisms was performed according to the standard operating protocol «Formic acid extraction» (Chebotar *et al.*, 2018). Identification, recording, processing, and analysis of mass spectra were carried out using the BioTyper RTC program. The accuracy of identification was judged by the value of the coefficient of coincidence (Score values: 2.000-3.000 – identification to the species; 1.999-1.700 – identification to the genus; 1.699-0 – identification failed) and the value of the categories (A – reliable identification up to the specie level, B – reliable identification up to the genus level, C – unreliable result).

The flexAnalysis program was used for profiling bacterial proteins, and the BioTyper MSP Dendrogram Creation Standard Method package included in the BioTyper 3.0 software and hardware complex was used to create an MSP dendrogram. Mass spectra from the Bruker database and the database of Academician I.N. Blokhina Nizhny Novgorod Scientific Research Institute of Epidemiology and Microbiology served as reference mass spectra. A total of 374 clones of three strains of bifidobacteria were identified.

Study of biochemical properties of strains

The biochemical properties of the strains were studied using standard API20A biochemical test systems (BioMerueux, France). To study the biochemical activity of bifidobacteria strains, 9 clones of each strain with a high Score were selected from cultures previously analyzed by mass spectrometry. Cultivation, sample preparation, identification of microorganisms, and interpretation of the obtained results were carried out according to the manufacturer's instructions; a total of 27 cultures were studied.

Whole genome sequencing

For the whole genome study, one culture of each strain with the highest Score and the studied biochemical profile was selected. Genomic DNA was isolated using a commercial kit QIAamp DNA Mini Kit «QIAGEN» (Germany); fragmentation was performed using a Covaris E210 ultrasonic fragmentation system «Applied Biosystems» (USA) according to the manufacturer's instructions. The mixture was purified, and fragments of 200–700 bp were selected using magnetic particles Agencourt AMPure beads «Beckman Coulter» (USA) and NEBNext Sizing Buffer «New England Biolabs» (USA). Libraries were prepared using the kit TrueSeq «Illumina Inc.» (USA); sequencing was performed on the platform MiSeq «Illumina Inc.» (USA). The original reads were processed by the Trimmomatic utility with standard parameters for Illumina. The processed reads were used for *de novo* genome assembly using the Spades, MIRA 4.0, and Newbler 2.6 programs.

Genome annotation

Genomes were annotated using the Prokka utility v. 1.11 (Seemann, 2014) and the RAST genomic server (http://rast.nmpdr.org). The search for determinants of antibiotic resistance and pathogenicity was carried out using software products presented on the website of the Center for Genomic Epidemiology (www.cge.cbs.dk): ResFinder 2.0, Pathogen Finder and PlasmidFinder (Seemann, 2014; Zankari *et al.*, 2012; Cosentino et el., 2013;

Carattoli *et al.*, 2014). The Bagel 4 program (van Heel *et al.*, 2018) was used to detect the genetic determinants responsible for the production of bacteriocins. Key enzymes responsible for the synthesis of neurometabolites were searched using the RAST genomic server (http://rast.nmpdr.org) (Aziz, 2008) and scientific literature data (Rossi *et al.*, 2011; Gabris *et al.*, 2015).

Construction of genomic maps

Genomic maps were constructed using the Proksee service (https://proksee.ca); hierarchical structures (dendrograms) constructed on the basis of SNP analysis of whole genome sequences of strains and describing their phylogenetic position were borrowed from the NCBI website.

Analysis of pan- and core genome of strains

To analyze the pan- and core genome, we used the PATO R package (Fernández-de-Bobadilla *et al.*, 2021), designed to analyze pangenomes of the same or different species (intraspecific/interspecific). PATO allows the analysis of population structure, phylogenetics, and horizontal gene transfer, using in each case a core genome (a set of genes common to all genomes), an accessory genome, or the entire genome. The files with format fna (fasta) containing amino acid sequences for the annotated proteins of the studied strains were used; next, redundancy-based filtering was performed, and identical strains (99.99% identity) were excluded from the analysis. The construction of ortholog clusters was carried out using MMSeq2 with standard parameters.

Study of the genetic diversity of strains

To study the level of genetic diversity of the strains, matrices of changes were constructed based on the results of the alignment of the amino acid sequences of the core genome proteins of the strains. The matrix was normalized by the total genome length (SNP/Megabase), then hierarchical clustering was performed. Matrix visualization is presented as a heat map; the color on the heat map represents the number of changes in the proteins of the core genome of a particular strain and varies from dark blue (0 changes) to red (30000 changes). Analysis of the metabolic potential of the strains was performed using the RAST genomic server; annotation of glycosyl hydrolases was carried out using the dbCAN2 web service, which allows automated annotation of carbohydrate-active enzymes (Zhang *et al.*, 2018).

Results

Morphological, tinctorial, and cultural properties of the studied strains of bifidobacteria

When cultivating on a plate medium (nutrient medium for determining and counting bifidobacteria, State Research Center for Applied Microbiology and Biotechnology, Obolensk) under anaerobic conditions, after 72 h of incubation at (38 ± 1) °C, *B. bifidum* 1 strain forms white, flat, with scalloped edge colonies with a convex center or white, flat, shiny colonies on the surface of the medium; *B. bifidum* 791 strain forms white, shiny colonies with a smooth edge, and *B. longum* 379 – convex, with even edges, white, shiny colonies with a smooth edge and creamy consistency. On a liquid medium (Bifidum medium, Obolensk), all strains were characterized by near tube bottom growth and colonies of «nail-shaped» form in the depth of the medium; Gram-stained smears showed gram-positive polymorphic rods with uneven edges and bifurcations, arranged as single cells or clusters in the form of Chinese characters.

Thus, it was found that despite long-term cultivation in laboratory conditions, all the studied strains have stable morphological, tinctorial, and cultural properties.

Results of strain identification and bacterial protein profiling using MALDI TOF mass spectrometry

Identification and analysis of mass spectra of 374 cultures of bifidobacteria were performed.

Strain identification using mass spectrometry was carried out using formic acid protein extraction according to the standard operating protocol, and the results are presented in Table 1.

Table 1

Results of identification of the studied strains of microorganisms using MALDI-TOF mass spectrometry

No	Strain	Number of cultures identified	Mass spectrometry result	
			Microorganism	Score values, category
1	*B.bifidum* 1	132	*B.bifidum*	From 1.878 B to 2.375 A
2	*B. bifidum* 791	118	*B.bifidum*	From 1.935 B to 2.472 A
3	*B. longum* 379	124	*B. longum*	From 1.848 B to 2.339 A

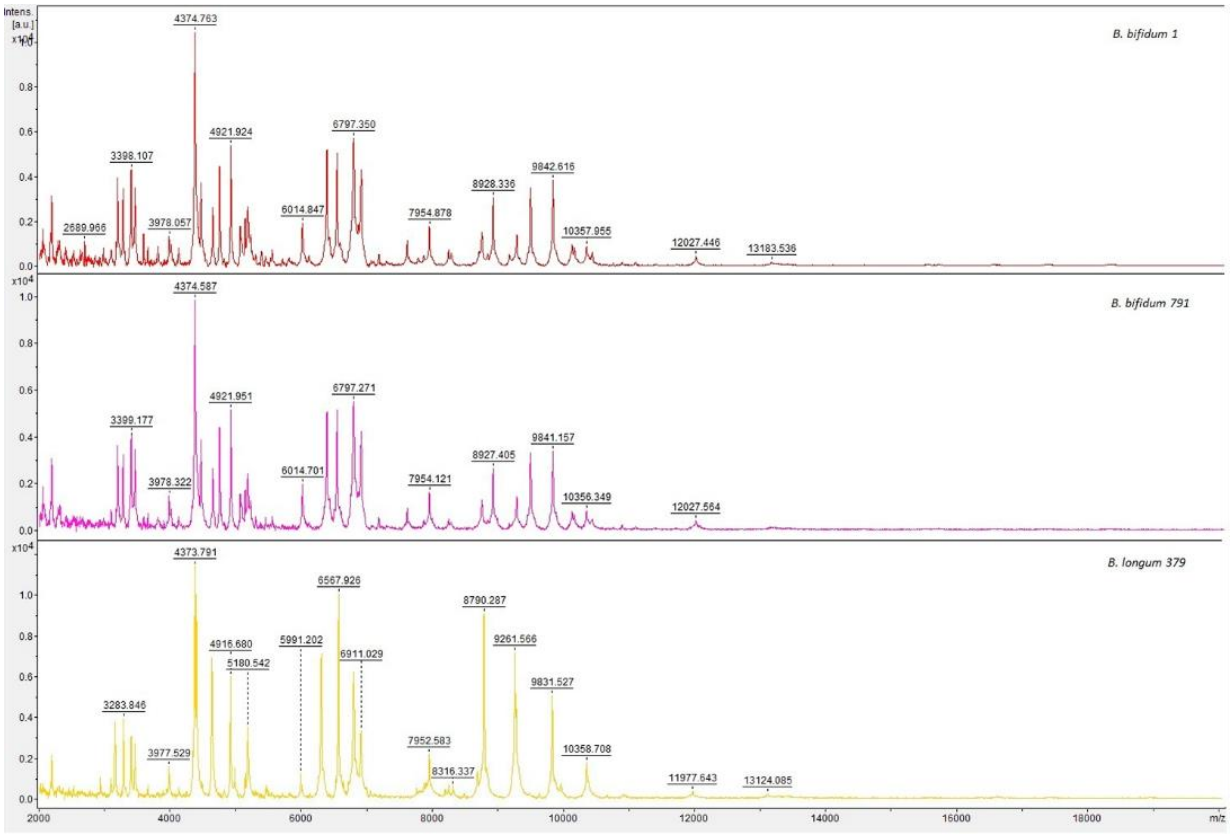

Fig. 1. MALDI mass spectra of strains of the genus *Bifidobacterium* using α-CHCA matrix

The MALDI TOF mass spectrometry principle is based on the measurement of the mass of an ionized substance. During MALDI ionization, singly charged ions are formed, i.e., one ion corresponds to one protein, so the method allows to identify a unique set of proteins for each of the studied microorganisms. When forming the mass spectrum, the resulting ions line up in ascending order of mass, while the intensity (height) of the peaks is not considered. When identifying microorganisms, the entire set of peaks is considered, not individual peaks.

Individual mass spectra were obtained for each bifidobacterial strain under study; typical MALDI mass spectra are shown in Fig. 1. Although the mass spectra were taken in the range of 2000-20000 m/z, the area from 2000 to 10000 m/z is visually the most informative.

The results of direct protein profiling can be obtained not only in graphical form (mass spectra) but also in tabular form (mass lists), which makes it possible to analyze all the presented peaks. Tables of the most reproducible mass peaks of three bifidobacteria strains were compiled as a result of this work (Table 2).

Table 2

List of the most reproducible mass peaks of the studied strains

B. bifidum 1	***B. bifidum 791***	***B. longum 379***
m/z	**m/z**	**m/z**
13183.536		
		13124.124
12027.446	12027.564	
		11977.643
10447.332	10445.155	
10357.955	10356.349	10358.708
10139.258	10138.777	
10121.511		
9842.616	9841.157	
		9831.527
9505.717	9502.255	
		9261.566
9288.645	9285. 021	
8928.336	8927.405	
		8790.287
8716.231		
		8316.336
		8193.591
7954.878	7954.121	7952.583
7615.687	7617.355	
6797.350	6797.271	6797.925
		6567.926
6544.080	6546.398	
6390.823	6390.442	
		6305.199
6014.847	6014.701	
		5991.202
5180.380	5179.643	5180.542
4921.924	4921.951	
		4916.680
		4631.920
		4395.924
4374.763	4374.587	4373.791
3978.057	3978.322	3977.529
3398.104	3399.177	
		3283.846
3273.694	3273.416	
		3153.084
2304.624	2304.219	
2186.407	2186.754	

A comparative analysis of 374 obtained mass spectra revealed that a number of peaks are common for all three strains – corresponding to m/z values of 10357, 7954, 6797, 5180, 4374, 3978. The lists of ionized protein masses, the so-called «strain markers», were determined based on the analysis of the mass spectra of 374 strain clones. The *B. bifidum* 791 strain is characterized by peaks corresponding to the following masses m/z: 12027, 10445, 10138,

9841, 9502, 9285, 8927, 6546, 6390, 6014, 4921, 3399, 3273, 2304, 2186; for strain *B. bifidum* 1 the following peaks are specific: 13183, 10121, 8716; for strain *B. longum* 379: 13124, 11977, 9831, 9261, 8790, 8316, 8193, 6567, 6305, 5991, 4916, 4631, 4395, 3283, 3153.

Results of studying the biochemical properties of strains

The studied bifidobacteria are strains-producers of probiotic medicines and foodstuffs. In accordance with the requirements of regulatory documents, their biochemical properties must be stable regardless of the duration of storage and cultivation methods and are subject to constant laboratory control.

To study the biochemical activity, 27 pure cultures of bifidobacteria with a high Score were selected from the strains analyzed earlier by mass spectrometry (Table 3).

9 clones of *B. bifidum* 1, *B. bifidum* 791, and *B. longum* 379 strains with Score values from 2.19 to 2.47 were selected for the study. It was found that all the studied strains consume glucose, disaccharides (lactose, sucrose, maltose). *B. longum* 379 has more pronounced saccharolytic activity; in addition to the substrates listed above, this strain is able to consume mannose, trisaccharide melezitose, and raffinose. All strains are unable to ferment cellobiose (4-β-glucoside-glucose), trehalose disaccharide, rhamnose deoxysaccharide, aldoses (xylose, arabinose), glucosides (salicin, esculin), polyhydric alcohols (glycerol, sorbitol, mannitol), gelatin.

It was found that the biochemical profiles of all the studied clones of probiotic strains are stable and correspond to the characteristics stated in the passports of the strains deposited in the State collection of pathogenic microorganisms, State collection of normal microflora microorganisms of G.N. Gabrichevsky Moscow Scientific Research Institute of Epidemiology and Microbiology, Russian national collection of industrial microorganisms (VKPM).

Results of whole genome sequencing of bacteria of the genus Bifidobacterium

One culture of each strain with known biochemical activity and the highest Score was selected for whole genome sequencing: *B. bifidum* 1 – 2.37; *B. bifidum* 791 – 2.47; *B. longum* 379 – 2.33. The main characteristics of the strain genomes are presented in Table 4.

Whole genome sequences of the strains were deposited in the international database GenBank: *B. bifidum* 1, GenBank: NDXI00000000 (2017), *B. bifidum* 791, GenBank: LKUR00000000 (2015), *B. longum* 379, GenBank: LKUQ00000000 (2015).

Whole-genome sequence analysis for pathogenicity and antibiotic resistance determinants

Using the PathogenFinder and PlasmidFinder services, we found that the genomes of all studied strains did not contain pathogenicity determinants and integrated plasmids. Analysis of whole-genome sequences for the presence of antibiotic resistance genes was performed using two services: the ResFinder 3.2 program and the RAST genomic server. According to ResFinder 3.2, no antibiotic resistance determinants were detected in the genomes of all strains. RAST allowed us to identify molecular efflux pumps of the MATE family; in addition, a cytoplasmic protein that protects the ribosome from tetracycline exposure (tetW) was identified in *B. longum* 379 strain.

Analysis of genomic maps of the strains

Genomic maps of strains were obtained (Fig. 2, 3, 4). The genomic map allows us to graphically represent and visually evaluate the structure and nature of the genome of a microorganism.

An analysis of the obtained genomic maps revealed that all three strains have regions that are characterized by an increased or decreased level of GC bases. The presence of such regions may indicate that the genes located within them could have been introduced into the genome by horizontal transfer (Pinevich, 2009). These regions contain genes encoding metabolic enzymes (glycosyltransferases, xylanase, peptidase, sialidase, etc.) and TerB protein responsible for the resistance of the microorganism to harmful environmental factors, in particular to heavy metals.

Table 3

Biochemical properties of bifidobacteria under study – strains of probiotics producers

Strain	IND	URE	GLU	MAN	LAC	SAC	MAL	SAL	XYL	ARA	GEL	ESC	GLY	CEL	MNE	MLZ	RAF	SOR	PHA	TRE	CAT
B. bifidum 1	–	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B. bifidum 791	–	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B. longum 379	–	–	+	–	+	+	+	–	–	–	–	–	–	–	+	+	+	–	–	–	–

* *Note:* IND – indole, URE – urease, GLU – glucose, MAN – mannitol, LAC – lactose, SAC – sucrose, MAL – maltose, SAL – salicin, XYL – xylose, ARA – arabinose, GEL – gelatin, ESC – esculin, GLY – glycerol, CEL – cellobiose, MNE – mannose, MLZ – melecytose, RAF – raffinose, SOR – sorbitol, PHA – rhamnose, TRE – trehalose, CAT – catalase.

Table 4

The main characteristics of the genomes of the studied strains of the genus *Bifidobacterium*

Strain	Number of contigs	Average coverage	Genome size, bp	GC composition, %	Amount of CDS	Number of tRNAs
B. bifidum 1, GenBank: NDXI00000000	13	385.0	2,198,027	62.7	1521	53
B. bifidum 791, GenBank: LKUR00000000	33	150.0	2,285,457	62.4	1769	52
B. longum 379, GenBank: LKUQ00000000	24	150.0	2,387,620	60.2	1903	71

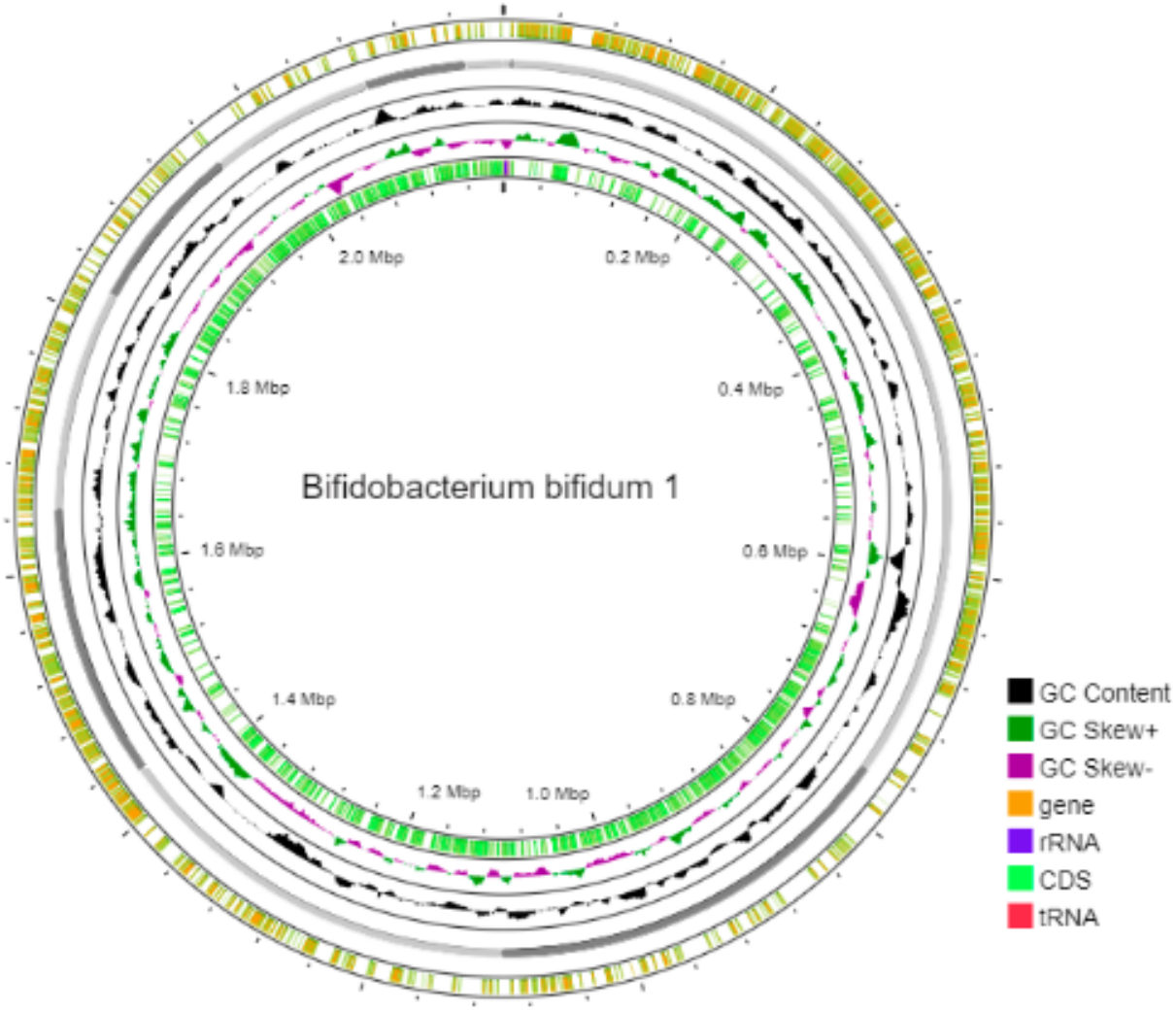

Fig. 2. Genomic map of *B. bifidum* 1 strain obtained using the Proksee service (https://proksee.ca). GC content – content of GC bases (%), GC skew plus – regions with a high content of GC, GC skew minus – regions with a low content of GC, gene – genes, rRNA – determinants encoding ribosomal RNA, CDS – coding region, tRNA – transport RNA

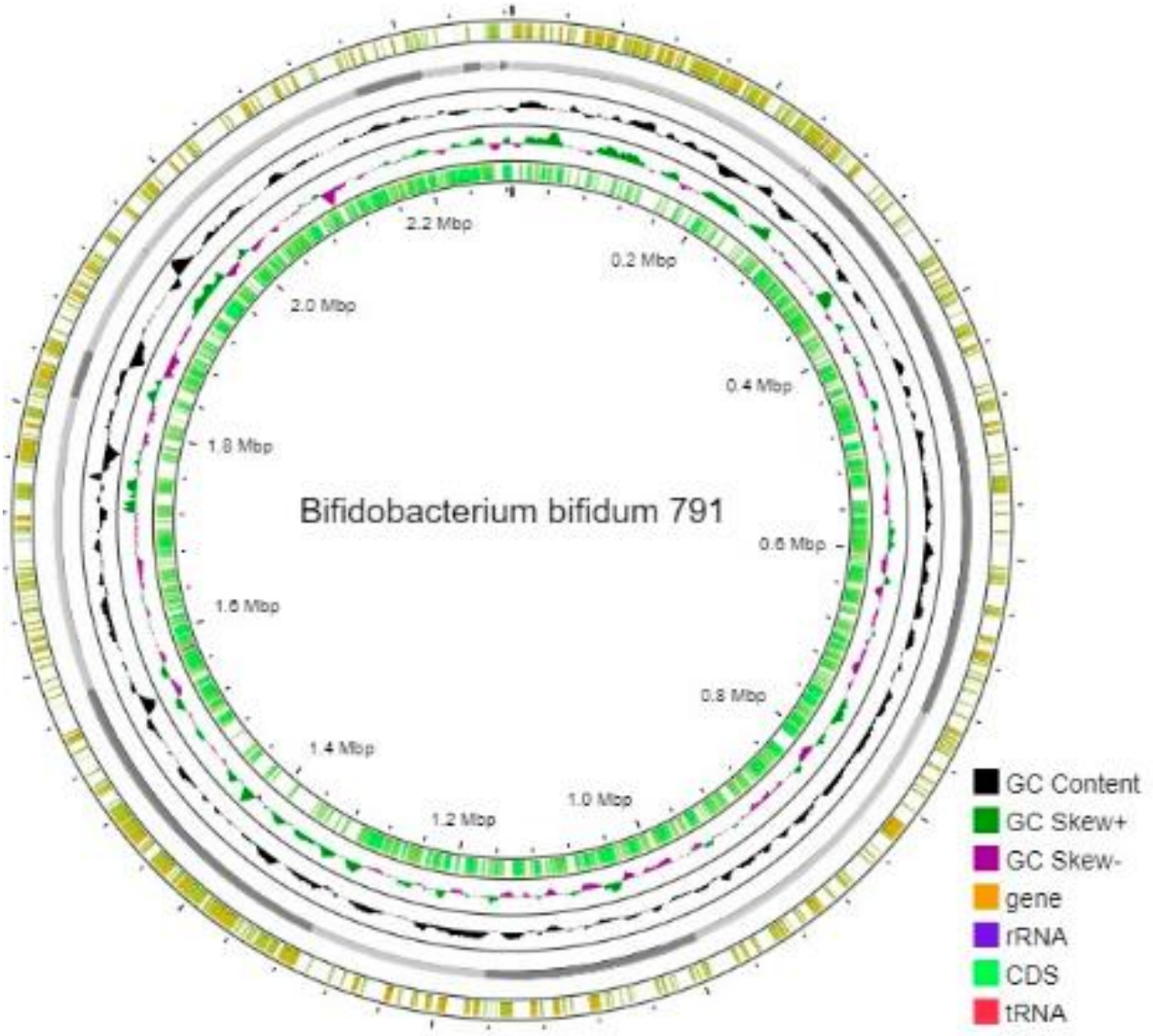

Fig. 3. Genomic map of *B. bifidum* 791 strain obtained using Proksee service (https://proksee.ca). GC content – content of GC bases (%), GC skew plus – regions with a high content of GC, GC skew minus – regions with a low content of GC, gene – genes, rRNA – determinants encoding ribosomal RNA

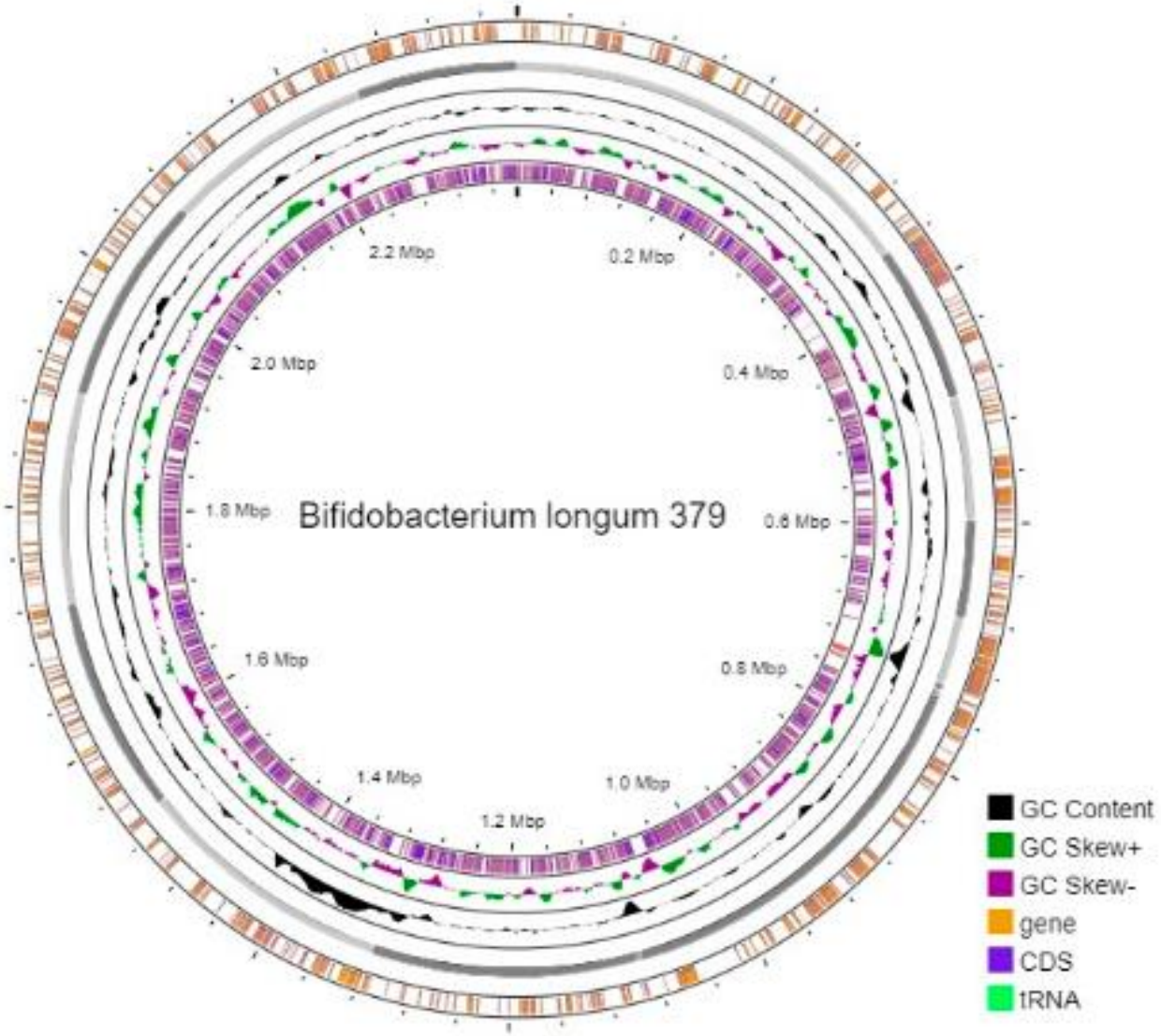

Fig. 4. Genomic map of *B. longum* 379 strain obtained using Proksee service (https://proksee.ca). GC content – content of GC bases (%), GC skew plus – regions with a high content of GC, GC skew minus – regions with a low content of GC, gene – genes, rRNA – determinants encoding ribosomal RNA, CDS – coding region, tRNA – transport RNA, CDS – coding region, tRNA – transport RNA

Analysis of the phylogenetic relationship of the strains under study

To understand the phylogenetic relationships between the studied and other strains of these species, the whole genome sequences of which are deposited in the international GenBank database, dendrograms constructed using the BLAST program were analyzed. To date, 178 whole genome sequences of representatives of the *B. bifidum* species and 670 whole genome sequences of the *B. longum* species are deposited in the GenBank database. Analysis of the dendrogram revealed that *B. bifidum* 791 strain is part of a cluster that includes 17 strains (Fig. 5A). The most phylogenetically close to B. *bifidum* 791 strains are *B. bifidum* ICIS-176 (GenBank: JAJHZI000000000.1, depositor – Institute of Cellular and Intracellular Symbiosis UrB RAS, Orenburg, Russia), *B. bifidum* VKPM Ac-1784 (GenBank: JACTOF000000000.1, depositor – Kurchatov Institute, Moscow, Russia), and *B. bifidum* ICIS-202 (GenBank: SSMS00000000.1, de positor – Institute of Cellular and Intracellular Symbiosis UrB RAS, Orenburg, Russia). All these strains were isolated from human feces and exhibit probiotic properties (Bukharin *et al.*, 2019; Korzhenkov*et al.*, 2020).

The *B. bifidum* 1 strain is part of a cluster that includes 16 strains (Fig. 5B).

Among the most phylogenetically close to *B. bifidum* 1 strain are *B. bifidum* NCTC13001 (GenBank Reference Sequence: NZ_LR134344.1, depositor – Wellcome Sanger Institute, UK) isolated from infant feces, *B. bifidum* JCM 1255 (depositor – Graduate School of Frontier Sciences, University of Tokyo, Japan), also isolated from infant feces (Morita *et al.*, 2019), and other variants of the *B. bifidum* JCM 1255 strain stored in various collections: *B. bifidum* ATCC 29251 (GenBank: AWSW00000000.1, depositor – Washington University Genome Sequencing Center, St. Louis, USA) and *B. bifidum* DSM 20456 (GenBank: JDUM00000000.1, depositor – School of Food Science and Engineering, China) (Vasiliev, 2017). In addition, the cluster of phylogenetically related strains includes *B. bifidum* UBBB-55 strain (GenBank: JADPYV000000000.1, depositor – Unique Biotech Limited, India; a probiotic strain with anticarcinogenic activity (Yenuganti *et al.*, 2021)), *B. bifidum* s-1 (GenBank: JACEIZ000000000.1, depositor – China Center of Industrial Culture Collection, China), *B. bifidum* BB-G90 (GenBank: JAGJCE000000000.1, depositor – Research and Development Center, China; a probiotic strain that is part of the probiotic to support athletes (Tavares-Silva *et al.*, 2021)), *B. bifidum* LMG 11041 (GenBank: JGYO00000000.1, depositor – Life Sciences, University of Parma, Italy; probiotic strain isolated from infant feces). Thus, *B. bifidum* 1 and B. *bifidum* 791 strains belong to the clusters of probiotic strains isolated from human feces.

Figure 6 demonstrates a fragment of the dendrogram containing a cluster of strains phylogenetically close to *B. longum* 379. According to the dendrogram, this strain is part of a cluster that unites 12 strains; the most phylogenetically close to it are *B. longum* ICIS-206 (GenBank Reference Sequence: NZ_JAJBBZ000000000.1, depositor – Institute of Cellular and Intracellular Symbiosis UrB RAS, Orenburg, Russia; a probiotic strain isolated from the intestines of a healthy person (Ivanova *et al.*, 2018)) and *Bifidobacterium longum* CCUG30698 (GenBank: CP011965.1, depositor – University College Cork, Ireland). It was found *that B. longum* 379 is a member of a cluster formed by strains of the subspecies *Bifidobacterium longum subsp. longum*: *B. longum subsp. longum* CCUG30698 (GenBank: CP011965.1, University College Cork, Ireland), *B. longum subsp. longum* KCTC 3421 (GenBank Reference Sequence: NZ_CP071590.1, College of Health Science, Korea University, Korea), *B. longum subsp. longum* 296B (GenBank Reference Sequence: NZ_MLZK00000000.1, Life Sciences, University of Parma, Italy), *B. longum subsp. longum* VKPM Ac-1635 (GenBank Reference Sequence: NZ_JACTOE000000000.1, Kurchatov Institute), *B. longum* DS18_3 (GenBank Reference Sequence: NZ_QDJB01000174.1, Department of Molecular Biology, USA), *B. longum subsp. longum* BL-G301 (GenBank Reference Sequence: NZ_JAFKPI000000000.1, Basic Research Department, Research and Development Center, China), *B. longum subsp. longum* MCC10103 (GenBank Reference Sequence: NZ_SHSW01000009.1, depositor – Next Generation Science Institute, Japan), which allows us to attribute it to this subspecies.

Fig. 5. Dendrogram of *B. bifidum* 1 and *B. bifidum* 791 strains obtained using BLAST. Genomic BLAST file was downloaded from GenBank database

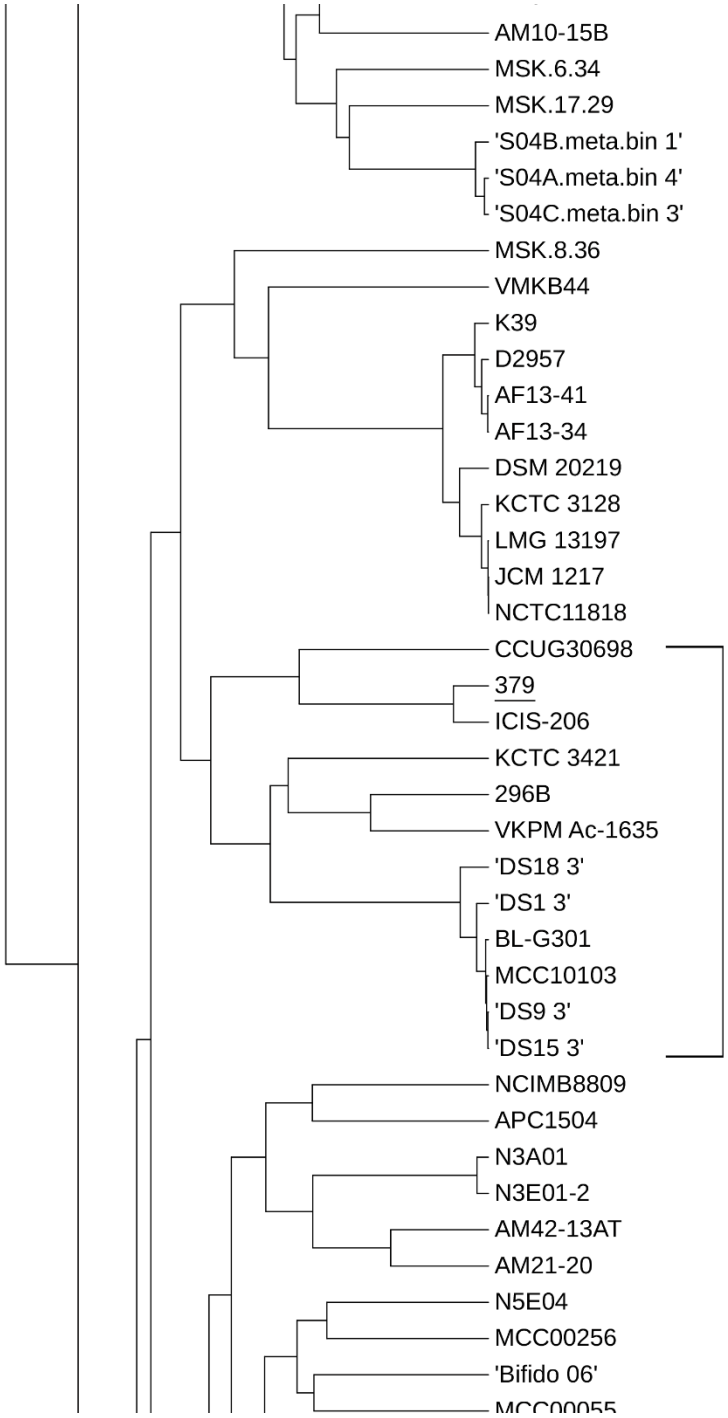

Fig. 6. Dendrogram of *B. longum* 379 strain obtained using BLAST. Genomic BLAST file was downloaded from GenBank database

Analysis of the level of genetic diversity of the strains

To assess the level of genetic diversity of *B. bifidum* and *B. longum* species, an analysis of pan- and core genomes of 695 strains of these species deposited in GenBank was carried out. Pangenome is the totality of all genes of the considered group of organisms (usually monophyletic), for which genetic diversity between closely related strains is possible. The core genome is genes present in all strains of the studied taxon.

To analyze the pan- and core genome of the *B. bifidum* species, graphs showing the dependence of the number of genes on the number of studied strains were constructed based on the genetic sequences of 95 strains (Fig. 7). It was found that the pangenome of *B. bifidum* contains 4952 protein-coding genes, while the core genome (genes present in > 95% of strains of the species) includes 1154 genes, and the accessory genome (genes present in < 95% strains) – 3798 genes. An analysis of the resulting graph allows us to conclude that the pangenome is open, which may indicate a significant role of horizontal gene transfer in the evolution of *B. bifidum.*

To analyze the level of genetic diversity of strains of the *B. longum* species, graphs showing the dependence of the number of genes on the number of studied strains were constructed based on the genetic sequences of 600 strains (Fig. 8).

It was found that the *B. longum* pangenome contains 15405 protein-coding genes, while the core genome (genes present in > 95% of strains of the species) includes 1082 genes, and the accessory genome (genes present in < 95% of strains) – 14323 genes. The pangenome of this species is also open, which may indicate a significant role of horizontal gene transfer in its evolution. Also noteworthy is the difference in the sizes of the accessory genomes of representatives of the *B. bifidum* and *B. longum* species, which may indicate a more pronounced metabolic activity of the *B. longum* strain.

Another method for assessing the phylogenetic position of strains is the method of constructing a matrix of changes (SNP) from the alignment of the amino acid sequences of strain core genome proteins with visualization in the form of a heat map (Fig. 9).

According to the color scale, species with similar protein sequences are colored dark blue; in the presence of dissimilar proteins, they have a shade from light blue to yellow. Analysis of the obtained heat map identified the strains with the closest sequences of proteins determined by the core genome genes: for *B. bifidum* 1 these are *B. bifidum* LMG 11041, *B. bifidum* NCTC13001, *B. bifidum* UBBB-55, *B. bifidum* s-1, *B. bifidum* BB-G90, the same strains, the phylogenetic relationship with which was found during the dendrogram analysis (Fig. 5). *B. bifidum* 791 strain deposited by us is a part of a unicolored cluster which includes *B. bifidum* ICIS-176, *B. bifidum* VKPM Ac-1784 strains, and the *B. bifidum* 791 strain of the same name, deposited by Institute of Cellular and Intracellular Symbiosis UrB RAS, Orenburg, Russia.

The analysis of the heat map constructed for strains of the *B. longum* species revealed that the studied *B. longum* 379 strain is included in a large cluster of strains with similar protein amino acid sequences; however, no strains with a high degree of identity were found (Fig. 10).

Thus, the pangenomes of both species are classified as open, which indicates a significant role of the process of horizontal gene transfer in the evolution of these species. The size of the *B. bifidum* pangenome was 4952 protein-coding genes, the core genome included 1154 genes; the size of the *B. longum* pan- and core genome was 15405 and 1082 genes, respectively. When analyzing the similarity of amino acid sequences of proteins encoded by the strains' core genome, it was found that *B. bifidum* 1 and *B. bifidum* 791 demonstrate a high degree of similarity with individual probiotic strains of this species. *B. longum* 379 is included in a large cluster that also contains a number of probiotic strains with similar amino acid sequences of proteins encoded by the core genome; however, no strains with a high degree of identity were found.

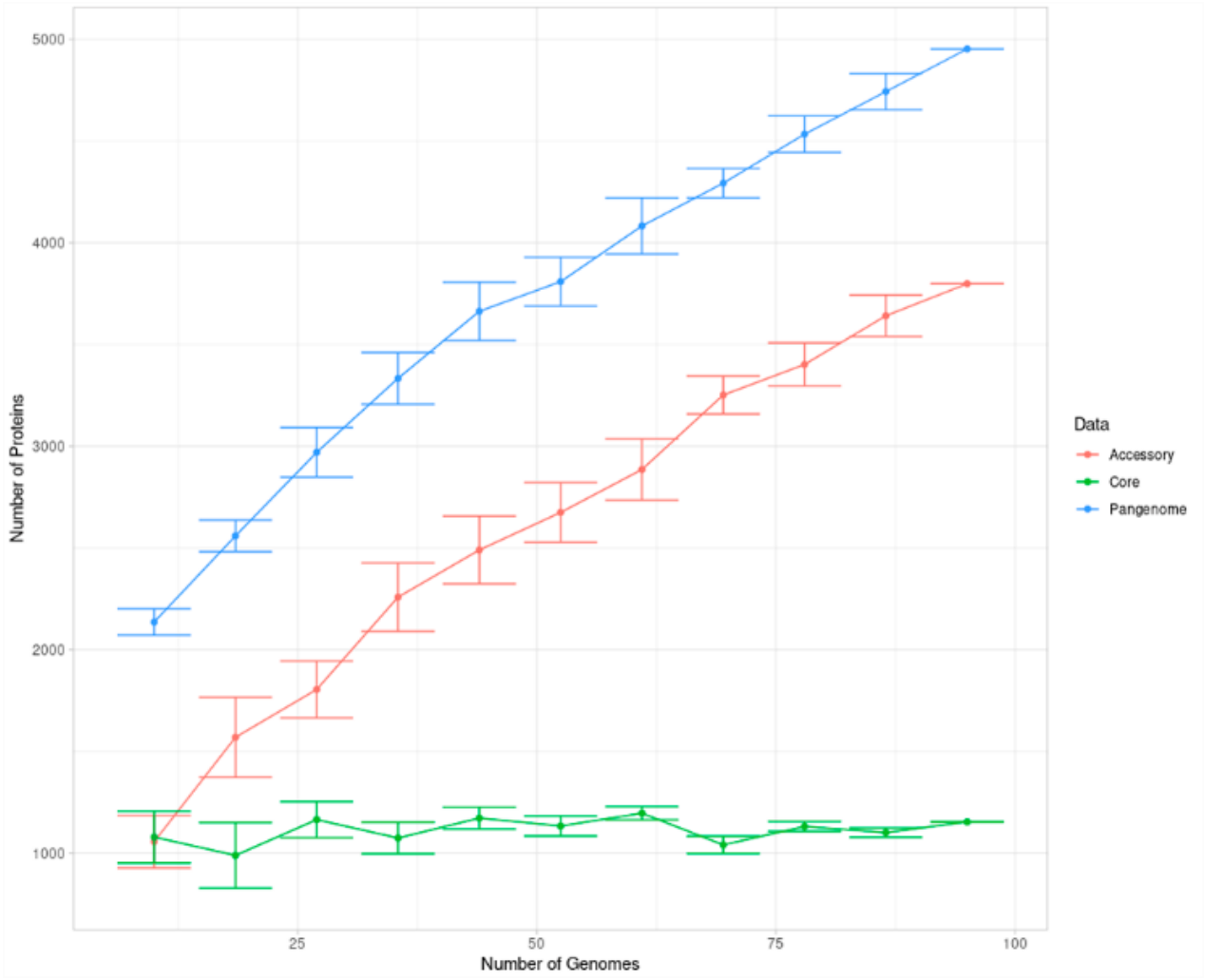

Fig. 7. Graph showing the concept of *B. bifidum* bacterial pangenome

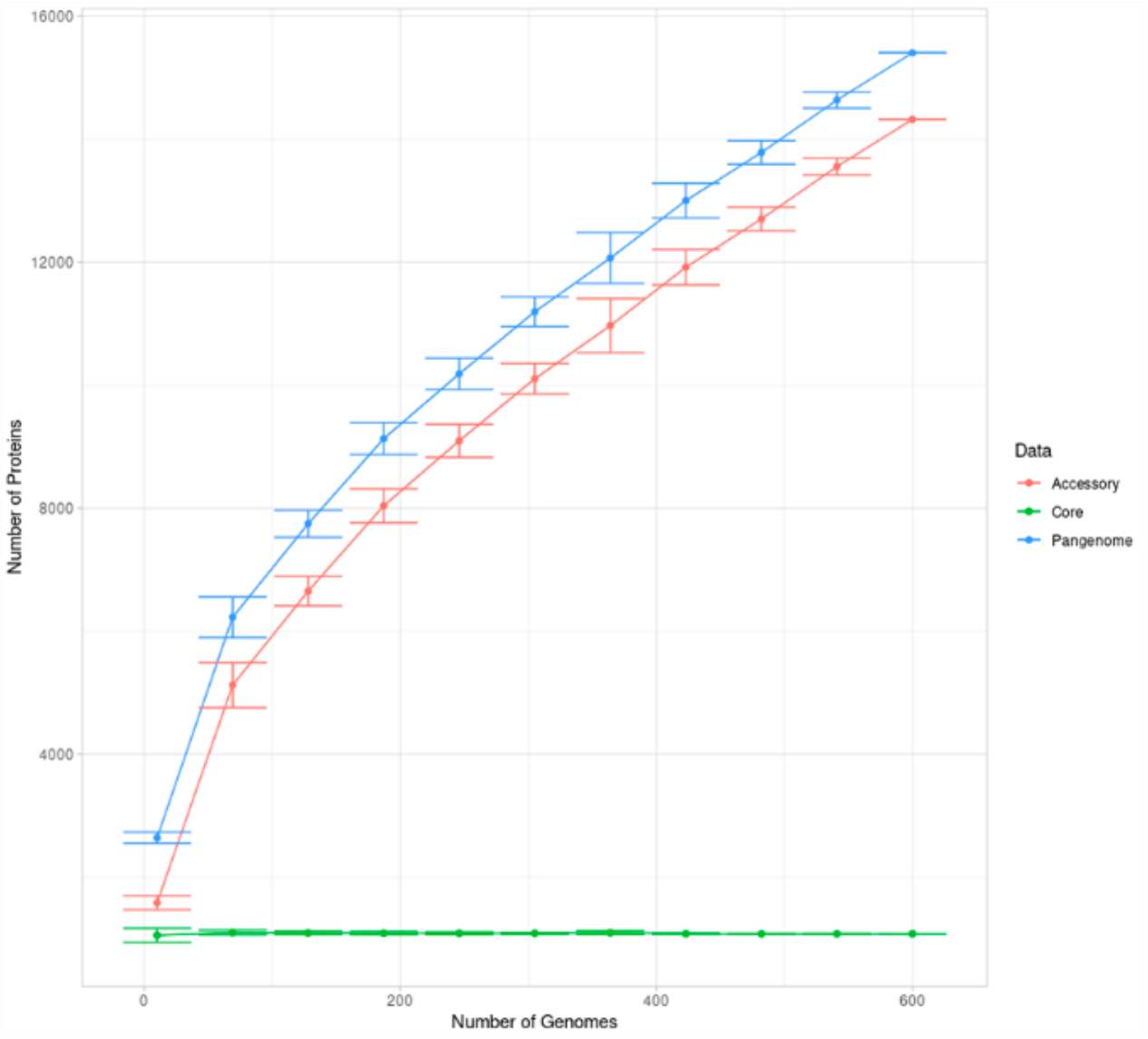

Fig. 8. Graph showing the concept of *B. longum* bacterial pangenome

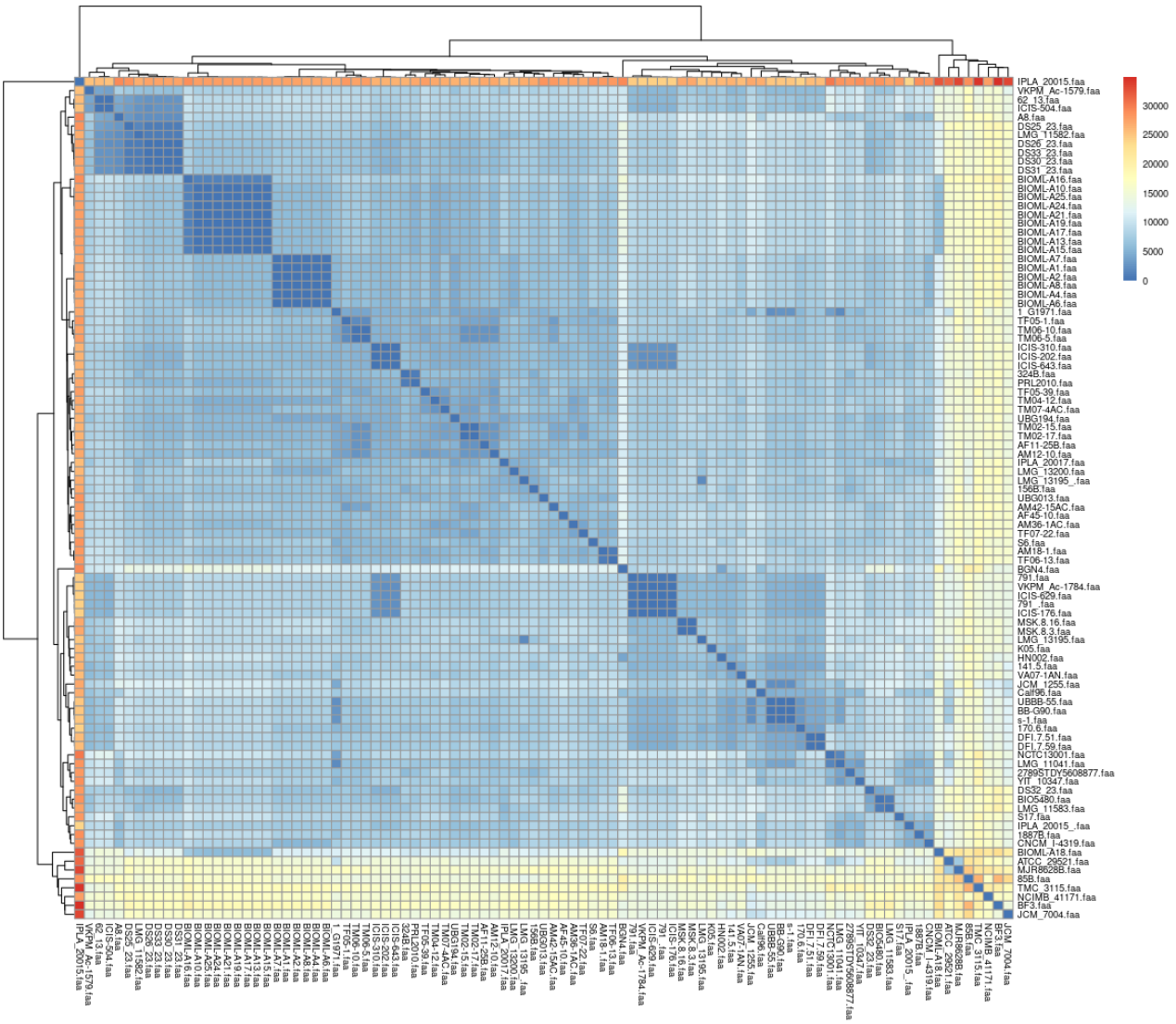

Fig. 9. Heat map representing the percentage of average amino acid sequence identity of the core genomes of 95 strains of *B. bifidum*. The color key represents the percentage of identity among the strains, strains with few changes (blue), and those with more changes (red). Strains are grouped into dendrograms based on the average values of the series

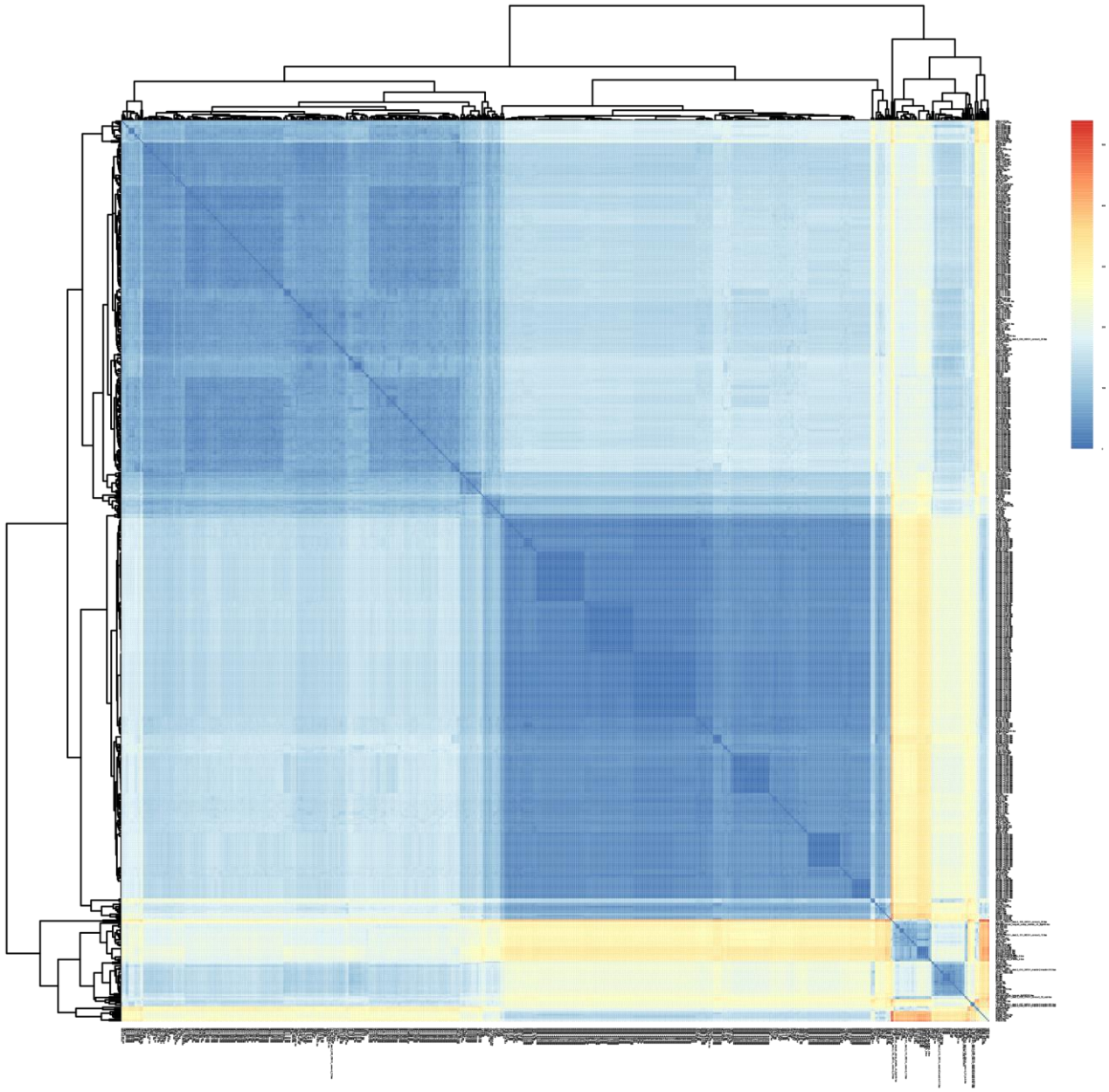

Fig. 10. Heat map representing the percentage of average amino acid sequence identity of the core genomes of 600 strains of *B. longum*. The color key represents the percentage of identity among the strains, strains with few changes (blue), and those with more changes (red). Strains are grouped into dendrograms based on the average values of the series

Analysis of the metabolic potential and glycosyl hydrolase genes of the strains

The obtained genomic sequences were analyzed using the RAST (Rapid Annotation using Subsystems Technology) genomic server (http://rast.nmpdr.org). It was found that in all the strains studied, the most widely represented are the subsystems of protein and sugar metabolism; the subsystems of sugar metabolism of all strains include determinants of the phosphoketolase pathway (fructose-6-phosphate phosphoketolase pathway), whose products are lactic, acetic acids and ethanol.

The protein and sugar metabolism subsystems of the *B. bifidum* 1 strain consist of 225 and 175 determinants, respectively (Table 5). The strain has a low ability to metabolize monosaccharides but is active against di-, oligosaccharides, and amino sugars: chitin and N-acetylglucosamine. Exopolysaccharide synthesis genes were not found; however, there are determinants responsible for the formation of sortase-dependent pili (SrtA, GenBank: PDH97100.1, PDH97310.1), AP surface protein, as well as cell wall lipoproteins (Lgt, GenBank: PDH98440.1 and LspA, GenBank: PDH98074.1).

The protein and sugar metabolism subsystems of the *B. bifidum* 791 strain consist of 207 and 150 determinants, respectively (Table 5). In terms of its saccharolytic and other properties, *B. bifidum* 791 strain is close to *B. bifidum* 1 strain. Thus, the genome of strain *B. bifidum* 791 contains almost no determinants of monosaccharide metabolism; however, there are genes responsible for the breakdown of more complex carbohydrates: di-, oligosaccharides, amino sugars, and starch. The genome contains genes encoding exopolysaccharide synthesis enzymes, including rhamnose synthesis genes located within contig 3 (GenBank LKUR01000023.1), determinants responsible for the formation of sortase-dependent pili (SrtA, GenBank: KYJ84870.1) and AP surface protein (GenBank: KYJ84871.1), as well as cell wall lipoproteins Lgt (GenBank: KYJ84380.1) and LspA (GenBank: KYJ85145.1).

The subsystems of protein and sugar metabolism of the *B. longum* 379 strain consist of 212 and 199 determinants, respectively (Table 5). The determinants responsible for the utilization of monosaccharides (xylose, ribose, arabinose), disaccharides (sucrose, maltose, lactose, raffinose, and phosphorylated oligosaccharide), amino sugars, and starch were found in the genome. Exopolysaccharide synthesis determinants are also presented: rhamnose synthesis genes located within contig 5 (GenBank: LKUQ01000020.1), capsular polysaccharide genes Wzb (tyrosine kinase) (GenBank: KYJ83195.1), Wzc (tyrosine phosphatase) KYJ83223.1, genes responsible for the formation of sortase-dependent pili – SrtA determinants (sortase A) (GenBank: KYJ83477.1) and AP (GenBank: KYJ83476.1), as well as lipoproteins – genes Lgt (GenBank: KYJ83617.1) and LspA (GenBank: KYJ77995.1).

Analysis of glycosyl hydrolase genes of the strains

The genome of bifidobacteria is specialized in the fermentation of a wide range of complex carbohydrates; therefore, a significant number of papers are devoted to the study of their glycosyl hydrolases (GH) – enzymes that catalyze the hydrolysis of glycosidic bonds in carbohydrate molecules, leading to the appearance of smaller molecules. The analysis of glycosyl hydrolases of the studied strains identified several groups of these enzymes. The first group was found in the genomes of all three strains: GH2, GH13 (enzymes that break α-glucosidic bonds, such as α-amylases), GH20 (β-hexosaminidase), GH3 (β-glucosidases, β-xylosidases, N-acetylhexominidase, which contribute to mucin glycan degradation), GH32 (invertase), GH36 (α-galactosidase), GH42 (β-galactosidase), GH43, GH51 (α-L-arabinofurosidase (plant fiber utilization)), GH7 (endo-β-1,4-arabinogalactan galactanase), GH101 (endo-α-N-acetylgalactosominidase (GMO and mucin degradation)), GH129 (α-N-acetylgalactosominase (mucin degradation)). The presence of these enzymes reflects the basic ability of the studied strains of bifidobacteria to consume galactooligosaccharides, including breast milk, plant fibers, and mucin glycans (Table 6).

Glycosyl hydrolases of the second group were found only in the genomes of *B. bifidum* 1 and *B. bifidum* 791: GH1, GH29 (α-fucosidases), GH110 (α-1,3-galactosidase), GH123 (β-N-acetylgalactose minidase, glycosphingolipid β-N-acetylgalactosominidase (degradation of mucin glycans)). The genome of *B. bifidum* 791 contains GH84 (hyaluronic glucosaminidase, β-N-acetyl hexosominidase), GH95 (α-1,2-L-fucosidase), GH33 (sialidase), enzymes also associated with the degradation of mucin glycans.

Glycosyl hydrolases of the third group were present only in the *B. longum* 379 genome: GH31 (α-D-xyloside xylohydrolase), GH5, GH30, GH12 (xylosidases), GH121, GH127, GH146 (3.2.1.185 β-L-arabinofuranosidase). All these enzymes belong to hemicellulases and are associated with the utilization of plant pol ysaccharides – plant fibers and mucus. Compared to *B. bifidum* 1 and *B. bifidum* 791, the genome of *B. longum* 379 also contains a larger number of enzymes of the GH13 groups – amylases (8) and GH43 – arabinofurazidases (10), which also play a crucial role in the breakdown and use of plant polysaccharides.

Table 5

Functional annotation of strains using RAST

Subsystem name, number of genes	Strains		
	B. bifidum 1	*B. bifidum* 791	*B. longum* 379
Carbohydrates	175	150	199
Protein metabolism	225	207	212
Amino Acid and Derivatives	225	198	207
DNA Metabolism	80	66	67
Fatty acids, Lipids end Isoprenoids	40	27	25
Cofactors, Vitamins, Prosthetic Groups, Pigments	77	73	96
Cell Wall	63	70	65

Table 6

Representatives of glycosyl hydrolase families in the genomes of the studied strains

Family name according to CAZy classification	The number of determinants encoding an enzyme in the strain genome; enzyme classification according to Enzyme Classification (EC)		
	B. bifidum 1	*B. bifidum* 791	*B. longum* 379
GH2	1 β-galactosidase (EC 3.2.1.23)	3 β-galactosidase (EC 3.2.1.23)	1 β-galactosidase (EC 3.2.1.23)
GH3	1 β-glucosidases (EC 3.2.1.21)	3 β-glucosidase (EC 3.2.1.21)	–
GH13	2 phosphate α-maltosyl-transferase (EC 2.4.99.16)	3 phosphate α-maltosyltransferase (EC 2.4.99.16) α-glucosidase (EC 3.2.1.20)	8 isomaltulose synthase oligo-α-glucosidase (EC 3.2.1.20) α-glucosidase (EC 3.2.1.20) glucodextranase (EC 3.2.1.68) isoamylase (EC 3.2.1.68)

Continuation of the table 6

Family name according to CAZy classification	The number of determinants encoding an enzyme in the strain genome; enzyme classification according to Enzyme Classification (EC)		
	B. bifidum 1	*B. bifidum* 791	*B. longum* 379
			4-α-glucanotransferase (EC 2.4.1.25) branching enzyme (EC 2.4.1.18) isomaltulose synthase (EC 5.4.99.11) starch synthase (malto-syl-transferring) (EC 2.4.99.16) sucrose phosphorylase (EC 2.4.1.7)
GH20 1	1 β-hexosaminidase (EC 3.2.1.52)	1 β-hexosaminidase (EC 3.2.1.52) lacto-N-biosidase mucine (EC 3.2.1.140)	1 β-hexosaminidase (EC 3.2.1.52)
GH29	1 dextranase (EC 3.2.1.11)	1 dextranase (EC 3.2.1.11)	–
GH30	–	–	1 endo-β-1,4-xylanase (EC 3.2.1.8);
GH31	–	–	2 α-D-xyloside xylohydrolase (EC 3.2.1.177)
GH32	1 invertase (EC 3.2.1.26);	1 invertase (EC 3.2.1.26);	2 invertase (EC 3.2.1.26);
GH33	-	1 sialidase (EC 3.2.1.18)	–
GH36	1 α-galactosidase (EC 3.2.1.22)	1 α-galactosidase (EC 3.2.1.22)	1 α-galactosidase (EC 3.2.1.22)
GH42)	1 (EC 3.2.1.23)	2 (EC 3.2.1.23)	2 (EC 3.2.1.23)
GH43	1 β-xylosidase (EC 3.2.1.37)	1 β-xylosidase (EC 3.2.1.37)	10 non-reducing end α-L-arabinofuranosidase (EC 3.2.1.37) xylan 1,4-β-xylosidase (EC 3.2.1.145) galactan 1,3-β-galactosidase (EC 3.2.1.8) endo-1,4-β-xylanase (EC 3.2.1.37)
GH51	2 endoglucanase (EC 3.2.1.4); endo-β-1,4-xylanase (EC 3.2.1.8); β-xylosidase	1 endoglucanase (EC 3.2.1.4)	5 α-L-arabinofuranosidase (EC 3.2.1.55)

Continuation of the table 6

Family name according to CAZy classification	**The number of determinants encoding an enzyme in the strain genome; enzyme classification according to Enzyme Classification (EC)**		
	B. bifidum 1	*B. bifidum* 791	*B. longum* 379
GH53	–	–	1 arabinogalactan endo-β-1,4-galactanase (EC 3.2.1.89)
GH77	1 amylomaltase or 4-α-glucanotransferase (EC 2.4.1.25)	1 amylomaltase or 4-α-glucanotransferase (EC 2.4.1.25)	2 arabinogalactan endo-β-1,4-galactanase (EC 2.4.1.25)
GH84	–	2 acetylhexosaminidase (EC 3.2.1.52)	–
GH95	–	1 α-1,2-L-fucosidase (EC 3.2.1.63)	–
GH101	1 endo-α-*N*-acetylgalactosaminidase (EC 3.2.1.97)	1 endo-α-*N*-acetylgalactosaminidase (EC 3.2.1.97)	1 endo-α-N-acetylgalactosaminidase (EC 3.2.1.97)
GH109	1 α-N-acetylgalactosaminidase (EC 3.2.1.49);	–	1 α-N-acetylgalactos-aminidase (EC 3.2.1.49);
GH110	1 α-1,3-galactosidase (EC 3.2.1.-)	1 α-1,3-galactosidase (EC 3.2.1.-)	–
GH112	1 lacto-N-biose phosphorylase or galacto-N-biose phosphorylase (EC 2.4.1.211);	2 lacto-N-biose phosphorylase or galacto-N-biose phosphorylase (EC 2.4.1.211);	1 lacto-N-biose phosphorylase or galacto-N-biose phosphorylase (EC 2.4.1.211);
GH121	–	–	1 β-L-arabinobiosidase (EC 3.2.1.-)
GH120	–	–	1 3.2.1.37 β-xylosidase (EC 3.2.1.37)
GH123	1 β-N-acetylgalactosaminidase (EC 3.2.1.53);	1 β-N-acetylgalactosaminidase (EC 3.2.1.53);	–
GH127	–	–	2 non-reducing end β-L-arabinofuranosidase (EC 3.2.1.185)

The end of the table 6

Family name according to CAZy classification	The number of determinants encoding an enzyme in the strain genome; enzyme classification according to Enzyme Classification (EC)		
	B. bifidum 1	*B. bifidum* 791	*B. longum* 379
GH129	1 α-N-acetylgalactosaminidase (EC 3.2.1.49)	1 α-N-acetylgalactosaminidase (EC 3.2.1.49)	1 α-N-acetylgalactosaminidase (EC 3.2.1.49)
GH136	lacto-N-biosidase (EC 3.2.1.140);	–	1 3.2.1.140
GH146	–	–	1 β-L-arabinofuranosidase (EC 3.2.1.185)

Analysis of the ability of strains to synthesize neurometabolites

Analysis of the genomes of the studied strains in the genomes of *B. bifidum* 791 and *B. longum* 379 revealed the key enzymes for the synthesis of neurometabolites – tryptophan and folic acid. Thus, the *B. bifidum* 791 genome contains genes that determine the synthesis of α and β subunits of tryptophan synthase (GenBank: KYJ84379.1, KYJ84378.1), an enzyme responsible for the synthesis of tryptophan, and dihydropteroate synthase (GenBank: KYJ84132.1), responsible for folic acid synthesis. The genome of *B. longum* 379 also contains tryptophan synthase, α and β subunits (GenBank: KYJ83618.1, KYJ83619.1) and dihydropteroate synthase (GenBank: KYJ81979.1).

Analysis of the ability of strains to synthesize bacteriocins

The genomes of all studied bifidobacteria were analyzed for the presence of genes that determine bacteriocins. The *B. bifidum* 791 genome (contig 28; GenBank: LKUR01000021.1) contains determinants responsible for the synthesis of class I lantibiotics: lasso peptide, a ribosomal-produced peptide, and bacteriocin flavucin (Fig.11).

Analysis of the operon responsible for the synthesis of the lasso peptide revealed that it consists of the gene responsible for the synthesis of the linear precursor (core peptide) and the *LasC* synthetase, consisting of 3 subunits, which determines the conformational structure of the final product – the transformation of the linear peptide into the lasso peptide, in which the C-terminal part of the molecule is «threaded» through the N-terminal macrolactam ring. The operon also includes genes for ABC transporters responsible for peptide secretion (Fig. 11A). The amino acid sequence of the *B. bifidum* 791 lasso peptide was determined: LIMKQYIAPTVETIASFREATNGLWFGK-YVDIGGAKAPFPWGSN.

Analysis of the similarity of protein sequences using BLAST showed that the *B. bifidum* 791 lasso peptide is highly similarity to other lasso peptides of probiotic strains of bifidobacteria (Fig. 12).

Flavucin is an antimicrobial peptide similar in structure to nisin, a bacteriocin synthesized by *Lactococcus lactis*. An analysis of the operon responsible for flavucin synthesis revealed that the gene responsible for the synthesis of the core protein is flanked by the *LanC* (Lantibiotic biosynthesis protein) modification determinant and several ABC transporters (Fig. 11C). The amino acid sequence of *B. bifidum* 791 flavucin was determined: MATFDLDTHVEETEG-DARPQITSKFMCTPGCPTGGLACFTSQC-TKGCSFTGGSKH.

When analyzing the similarity of protein sequences using BLAST, it was found that *B. bifidum* 791 flavucin has a high similarity with other bacteriocins of the lantibiotic class – gallidermin and nisin (Fig. 13).

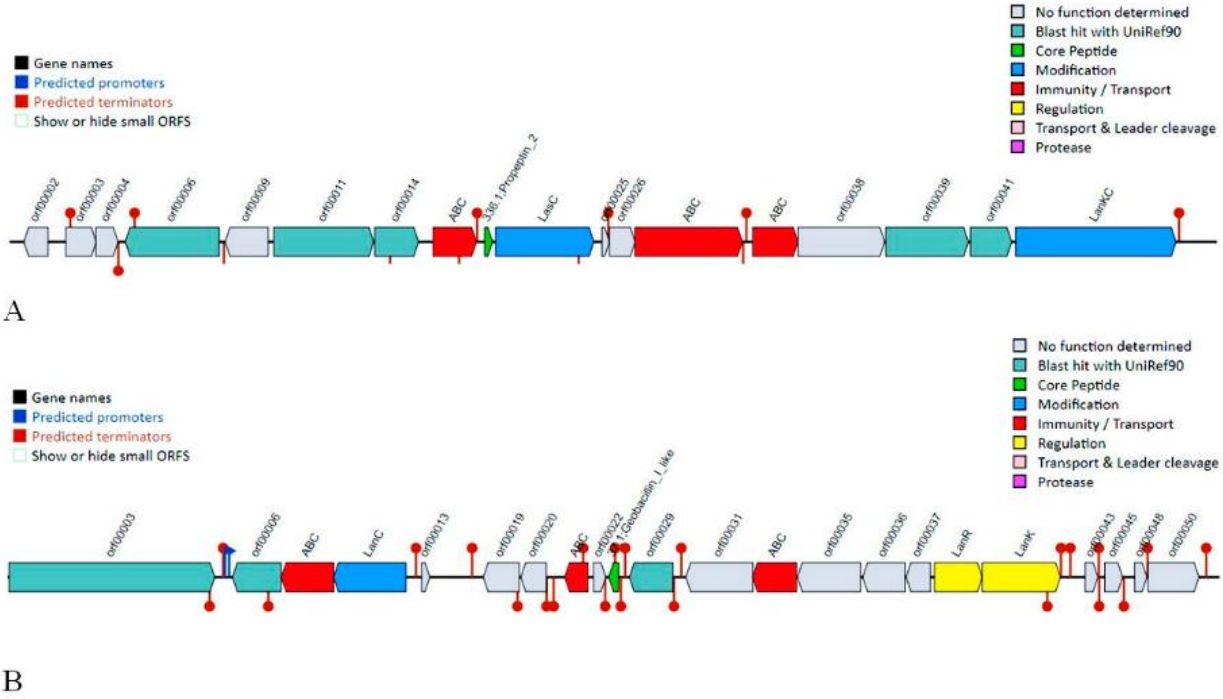

Fig. 11. Operons responsible for the synthesis of the lasso peptide (A) and flavucin (B) of *B. bifidum* 791 (data obtained using the BAGEL 4.0 genomic server)

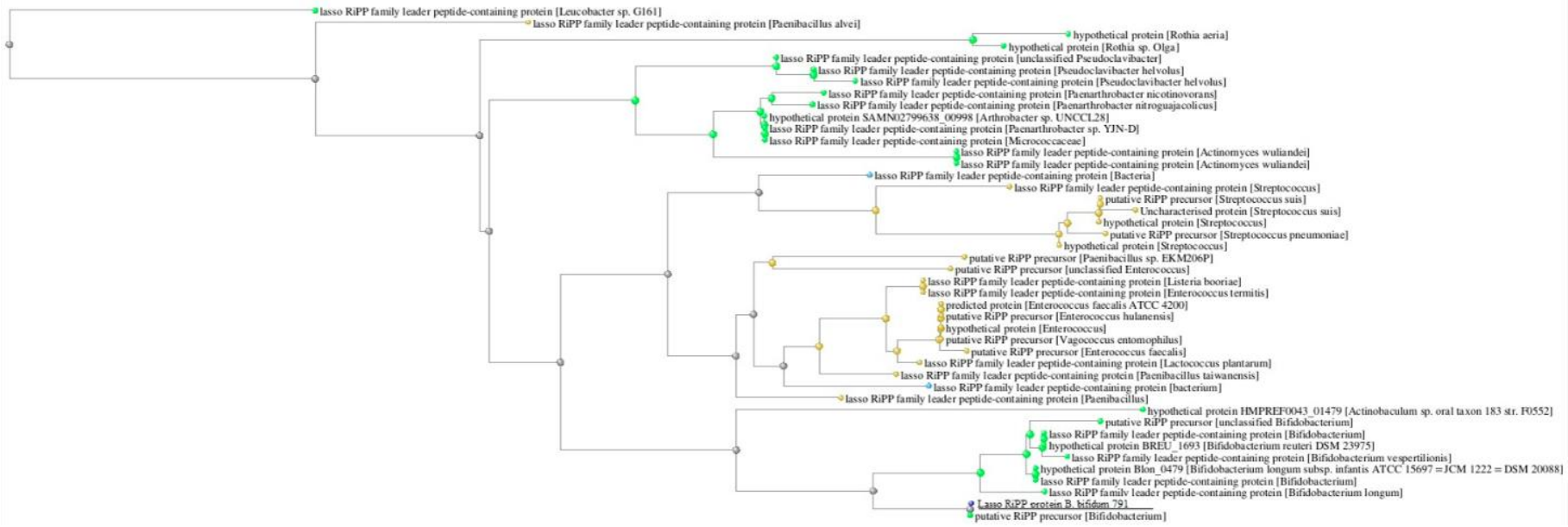

Fig. 12. Phylogenetic tree of amino acid sequences of lasso peptides of bacteria of the genus *Bifidobacterium* using the BLAST program

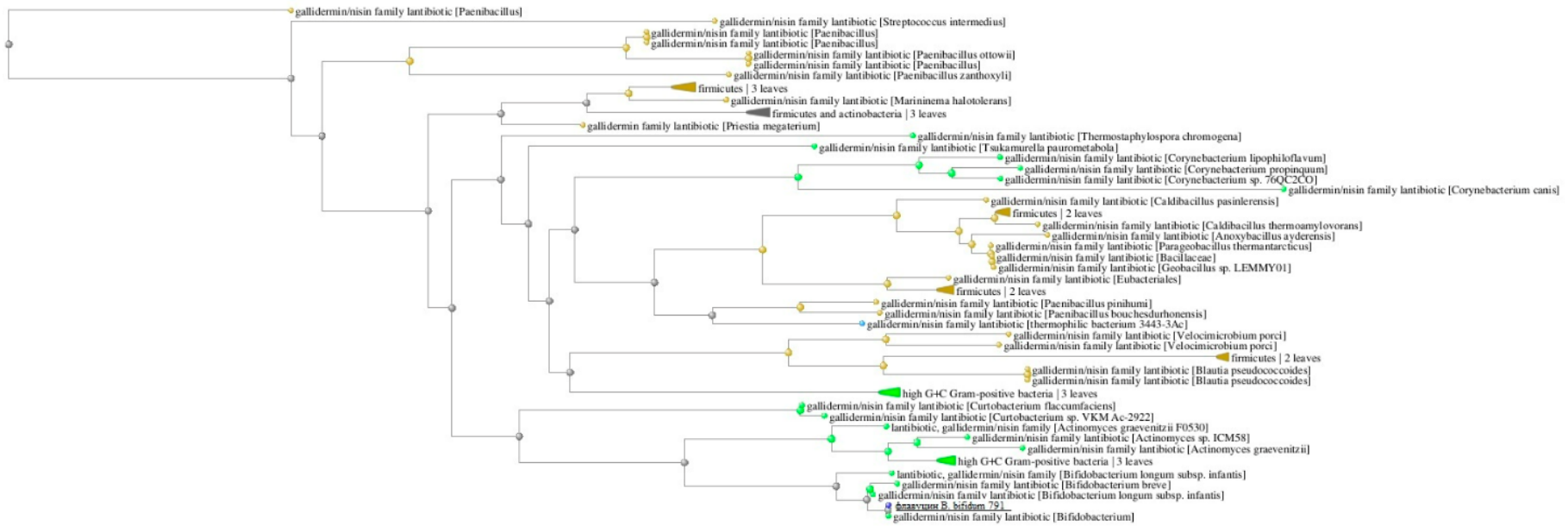

Fig. 13. Phylogenetic tree of amino acid sequences of lantibiotics of bacteria of the genus *Bifidobacterium* using the BLAST program

Discussion

It was found that probiotic bifidobacteria *Bifidobacterium bifidum* 1, *Bifidobacterium bifidum* 791, *Bifidobacterium longum* 379 have morphological, tinctorial, cultural, and stable biochemical properties typical for these strains. For the first time, direct profiling of bacterial proteins using MALDI TOF mass spectrometry revealed a list of masses of ionized proteins (peaks) typical for each of the three strains. The *B. bifidum* 791 strain is characterized by peaks corresponding to the following masses m/z: 12027, 10445, 10138, 9841, 9502, 9285, 8927, 6546, 6390, 6014, 4921, 3399, 3273, 2304, 2186; for strain *B. bifidum* 1 the following peaks are specific: 13183, 10121, 8716; for strain *B. longum* 379: 13124, 11977, 9831, 9261, 8790, 8316, 8193, 6567, 6305, 5991, 4916, 4631, 4395, 3283, 3153.

As a result of the analysis of whole genome sequences of bifidobacteria, it was found that the genomes of *B. bifidum* 1, *B. bifidum* 791, and *B. longum* 379 strains do not contain pathogenicity genes and integrated plasmids. Efflux pumps of the MATE family are antiporter membrane transport proteins that export drug compounds from the bacterial cell and do not belong to antibiotic resistance determinants. The *tetW* gene encoding a protein that protects the ribosome from the action of tetracycline is located within the sixth contig (LKUQ01000021.1); an analysis of its genomic context showed that this gene is not associated with the determinants responsible for the mobile transfer, and therefore does not pose a threat in terms of transmission.

The analysis of genomic maps of strains revealed that genes associated with parallel transfer encode metabolic enzymes (glycosyltransferases, xylanase, etc.), proteins responsible for resistance to harmful environmental factors, such as heavy metals (tellurite resistance protein, TerB) and proteins with an unknown functional role (hypothetical protein). The analysis of phylogenetic relationships of *B. bifidum* 1, *B. bifidum* 791, and *B. longum* 379 with 848 strains of these species, whole genome sequences are deposited in GenBank, revealed that the studied strains are part of phylogenetic clusters formed by probiotic strains, including foreign strains-producers of probiotic medicines and foodstuffs.

Pan- and core genomes of all *B. bifidum* (n = 95) and *B. longum* (n = 600) strains presented in GenBank were analyzed by the number of protein-coding genes. The pangenome of *B. bifidum* contains 4952 genes, the core genome – 1154 genes; the pangenome and core genome of *B. longum* contain 15405 and 1082 genes, respectively. The pangenomes of both species are classified as open, indicating a significant role of horizontal gene transfer in the evolution of these species. An analysis of the similarity of the amino acid sequences of proteins encoded by the core genome of the strains revealed that *B. bifidum* 1 and *B. bifidum* 791 demonstrate a high degree of similarity with individual probiotic strains of this species, including probiotic-producing strains. *B. longum* 379 is included in a large cluster that also contains a number of probiotic strains with similar amino acid sequences of proteins encoded by the core genome; however, strains with a high degree of identity were not found.

Analysis of the genetically determined metabolic potential of *B. bifidum* 1, *B. bifidum* 791, and *B. longum* 379 allowed us to reveal that the subsystems of protein and sugar metabolism are the most widely represented in them. It was shown that all strains possess determinants of the phosphoketolase pathway and are able to consume di-, oligosaccharides, and amino sugars. The products of active carbohydrate breakdown are lactic, acetic acids, and ethanol, which are among the most important biologically active compounds that provide slight acidification of the intestinal cavity, promote better absorption of electrolytes and inhibit the growth of pathogenic and opportunistic microorganisms. A high level of lactate stimulates the protrusion of macrophages (penetration of their processes from the mucosa into the intestinal cavity) and determines the immunoregulatory properties of bifidobacteria, while acetate, a valuable short-chain fatty acid, performs important energy tasks by being absorbed into the bloodstream and entering the cells of various organs and tissues, reduces the level of toxic metabolites and

carcinogens, normalizes the motility of the gastrointestinal tract, reduces the formation of ketones (Morita *et al.*, 2019; Kornienko, 2016).

The genome of bifidobacteria is specialized in the fermentation of a wide range of complex carbohydrates; therefore, a significant number of papers are devoted to the study of their glycosyl hydrolases, enzymes that catalyze the hydrolysis of glycosidic bonds in carbohydrate molecules, leading to the appearance of smaller molecules. Glycosyl hydrolases (GH) ensure the utilization of disaccharides, plant polysaccharides, breast milk oligosaccharides, and glycans of intestinal mucus, and statistically significant differences in the spectrum of these enzymes in different species of the genus *Bifidobacterium* were revealed, which reflects the specifics of their adaptation to the ecological niche occupied – the human colon (Schell *et al.*, 2002; Turroni *et al.*, 2010; Chaplin, 2015; Morita *et al.*, 2019; Fushinobu *et al.*, 2021; Kelly *et al.*, 2021). Analysis of the glycosyl hydrolase spectrum revealed that *B. bifidum* 1 and *B. bifidum* 791 strains have a pronounced ability to consume glycans of intestinal mucus. Their genomes contain enzymes for the degradation of mucin oligosaccharides: fucosidases, β-N-acetylgalactosaminidase, lacto-N-biosidase, hyaluronoglucosaminidase. A complex of specialized enzymes that allow efficient utilization of plant glycans, including xylo- and arabino-oligosaccharides, was identified in the genome of *B. longum* 379. The results obtained reflect the adaptation of these strains to the conditions of existence under a deficiency of simple carbohydrates in the lower intestine. Such a «distinction» in the choice of carbohydrate sources allows these strains of bifidobacteria to effectively coexist *in vivo*, avoiding competition for nutrient substrates. In addition, the complex of active bacterial hydrolases ensures the implementation of the probiotic properties of the strains: colonization resistance, antagonistic activity against the PM and OM, active symbiotic digestion, and, consequently, the most efficient absorption of food by the macroorganism.

It was also shown that *B. longum* 379 and *B. bifidum* 791 strains contain in the genome determinants of the synthesis of exopolysaccharides, sortase-dependent pili, and cell wall lipoproteins (LpAs). Exopolysaccharides of probiotic bacteria, sortase-dependent pili, and lipoproteins can physically prevent the adhesion of viruses; in addition, exopolysaccharides of bifidobacteria protect cells from aggressive secretions of the gastrointestinal tract, have a pronounced antibacterial effect, and are also used by other members of the intestinal microbiota as a nutrient substrate (Ermolenko *et al.*, 2003; El Kfoury *et al.*, 2017; Kang *et al.*, 2015).

Determinants encoding enzymes of tryptophan and folic acid synthesis, the most important neurometabolites with a pronounced thymoleptic effect (correction of increased anxiety, depression, and cognitive decline), were found in the genomes of the strains under study. Previously, researchers have already obtained evidence of the ability of bifidobacteria to increase the level of tryptophan in the blood of laboratory animals, to alleviate their condition under stress, that is, to have a thymoleptic effect (Oleskin & Shenderov, 2016). Tryptophan produced by bacteria and the tryptamine formed as a result of its decarboxylation can be delivered with the bloodstream to the brain and act as precursors of monoamine neurotransmitters, including serotonin (Oleskin & Shenderov, 2019). Serotonin deficiency causes pronounced brain disorders such as cognitive decline, increased anxiety, and depression.

Recently, bacteriocins synthesized by representatives of the normal human microbiota and probiotic-producing strains, including bacteria of the *Bifidobacterium* genus, have attracted the attention of researchers. Bacteriocins are peptides with broad-spectrum antimicrobial activity; the ability to synthesize them is strain-specific (Zaslavskaya *et al.*, 2019). It is known that strains capable of synthesizing bacteriocins have an ecological advantage over other strains under *in vivo* conditions, and bacteriocins of strains of normal microbiota are involved in the formation of "joint immunity" of the macroorganism (Zaslavskaya *et al.*, 2019). In the *B. bifidum* 791 genome, genes responsible for the synthesis of the lasso peptide and flavucin, bacteriocins belonging to class I lantibiotics with a

wide spectrum of antimicrobial activity, which was previously proven empirically, were found (Solovyeva *et al.*, 2012; Novik, 2006). Lantibiotics are antimicrobial substances containing the typical thioether amino acids lanthionine or methyllanthionine; most lantibiotics are synthesized on ribosomes and undergo post-translational modification. According to modern scientific data, lasso peptides have a wide range of antimicrobial and antiviral activity; they are active against bacteria of the genera *Salmonella, Shigella, E. coli, P. aeruginosa*, etc. The activity of lasso peptides against viruses is associated with the blocking of viral enzymes and receptors (van Heel *et al.*, 2016; Lu *et al.*, 2021; Tiwari *et al.*, 2020; Alvarez-Sieiro *et al.*, 2016; Maksimov *et al.*, 2012; Cheng & Hua, 2020). Flavucin is also known to have a wide spectrum of antimicrobial activity; there is data on the ability of structurally similar bacteriocins to inhibit all stages of viral replication, block their receptors, and prevent adsorption on eukaryotic cells (Lu *et al.*, 2021). The presence of lasso peptide and flavucin synthesis determinants in the genome of *B. bifidum* 791 strain is associated with its activity against the epidemic influenza A/Lipetsk/1V/2018 virus (H1N1) and the highly pathogenic avian influenza A/common gull/Saratov/1676/2018 virus (H5N6) (Soloveva *et al.*, 2021; Tochilina *et al.*, 2022).

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