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NEW PRODUCERS OF PREBIOTIC CARBOHYDRATES ISOLATED FROM BULGARIAN YOGURT

S U M M A R Y

of a PhD dissertation, Professional field 4.3. "Biological Sciences", scientific specialty "Microbiology"

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This dissertation was developed in the Department of General Microbiology, Laboratory of Microbial Genetics, Institute of Microbiology "Stefan Angelov" BAS. The dissertation work comprises 121 pages and is accompanied by 13 tables and 27 figures. The bibliography includes 218 literary sources. The dissertation work was discussed and directed for defense at a meeting of the National Seminar on General Microbiology of the Institute of Microbiology "Stefan Angelov", BAS, on 16 September 2025.

The defense of the dissertation will take place at an open meeting of the Scientific Jury on .....year. at ..... h in the hall of the Institute of Microbiology "Stefan Angelov", BAS, "Acad. G. Bonchev", bl. 26, Sofia. The Materials for the defense are available in room 218 (Clerical Office) at the Institute of Microbiology "Stefan Angelov", BAS, ul. "Acad. G. Bonchev, bl. 26, Sofia, and are published in accordance with the Regulations for the Application of the Law on the Development of the Academic Staff in the Republic of Bulgaria (LASRB), Bulgaria, at www.microbio.bas.bg.

List of abbreviations

LAB - Lactic acid bacteria
GIT - Gastrointestinal tract
GOS - Galactooligosaccharides
lacZ - Gene encoding the enzyme beta-galactosidase
CFU/ml - Colony forming units/ml
EC number - Enzyme Commission number
SDS - Sodium dodecyl sulfate
PAGE - Polyacrylamide gel electrophoresis
DNA - Deoxyribonucleic acid
RNA - Ribonucleic acid
mRNA - Messenger ribonucleic acid
bp - Base pair
GH - Glycoside hydrolase
DP - Degree of polymerization
ATCC - American Type Culture Collection
DSMZ - German Collection of Microorganisms and Cell Cultures

Introduction

Fermented foods have occupied an important place in human food culture since ancient times and continue to be the subject of intensive research in the field of nutrigenomics, microbiology and biotechnology. Of particular importance is lactic acid fermentation, which not only preserves foods, but also enriches their nutritional value, biological activity and safety.

Bulgarian yogurt is an emblematic product with international recognition, the uniqueness of which is due to the specific microflora - Lactobacillus delbrueckii ssp. bulgaricus and Streptococcus thermophilus, as well as the accompanying autochthonous microbial communities. These microorganisms not only participate in the fermentation process but also synthesize bioactive compounds with a prebiotic effect. Among them, galactooligosaccharides (GOS) - carbohydrates with proven prebiotic potential, which stimulate the growth of beneficial bacteria in the gastrointestinal tract and improve human health status, occupy a special place.

The present dissertation is dedicated to the study of new producers of GOS, isolated from traditional Bulgarian yogurts, the heterologous expression of the gene responsible for this and the biochemical characterization of the enzyme β-galactosidase. The high activity and unique properties of the L. bulgaricus enzyme allow for the development of a successful biotechnological process for the synthesis of GOS.

I. **The literature review** in the dissertation examines: the biology of lactic acid bacteria, Bulgarian fermented milk products, the structure and properties of GOP, the industrial application of oligosaccharides.

LAB are gram-positive microorganisms that metabolize carbohydrates to lactic acid. They include the genera *Lactobacillus, Streptococcus, Lactococcus, Leuconostoc*, and *Pediococcus*, among others. *L. delbrueckii* ssp. *bulgaricus*, occupies a special place and together with *S. thermophilus* forms a symbiotic starter culture for yogurt. This symbiosis determines the taste, aroma, consistency, and beneficial properties of the product. The review presents rich historical and technological information about yogurt, white brine cheese, yellow cheese, katyk, kefir, koumiss, and skimmed milk. They are not only foods, but also cultural heritage, carriers of diverse microbiota and bioactive substances. LAB synthesize organic acids, bacteriocins, exopolysaccharides, B vitamins, antioxidants, and antihypertensive peptides. They are associated with the probiotic and postbiotic potential of dairy products.

Galactooligosaccharides (GOS): carbohydrates made up of a glucose molecule linked to 2–8 galactose units. They act as prebiotics, stimulating the growth of bifidobacteria and lactobacilli in the colon. They have been shown to have a positive effect on mineral absorption, lipid metabolism, and immune response. The biosynthesis of GOS is catalyzed by β-galactosidases capable of performing transgalactosylation.

GOS are used in baby foods, probiotic supplements, and functional products. Modern biotechnology is looking for new enzymes with high activity and stability, which makes Bulgarian yogurt strains particularly valuable as a source of biocatalysts.

II. AIM AND OBJECTIVES

The dissertation aims to molecularly characterize the *lacZ* gene and β-galactosidase from the Bulgarian strain *Lactobacillus delbrueckii* subsp. *bulgaricus*, with a view to optimizing the in vitro synthesis of prebiotic galactooligosaccharides (GOS).

The implementation of the aim is related to the sequential experimental work on the following

OBJECTIVES:

1. To collect samples of homemade yogurts from different geographical regions of Bulgaria and to analyze the microbiota by metagenomic sequencing.

2. To identify and compare the species of lactic acid bacteria (LAC) and to evaluate the specific ratios of L. bulgaricus and S. thermophilus in yogurts.

3. To establish the presence of concomitant, atypical or probiotically significant strains and possible contaminants.

4. To sequence the lacZ gene from L. bulgaricus strain 43 and to analyze the molecular structure of the β-galactosidase enzyme encoded by this gene.

5. To carry out heterologous expression of lacZ in E. coli and to study the activity and biochemical characteristics of the β-galactosidase enzyme (pH optima, temperature, influence of metal ions).

6. To evaluate the potential of the new β-galactosidase for industrial production of GOS.

III. MATERIALS AND METHODS

1. Microbiological methods

Collection of yogurt samples

Isolation of LAB strains

Other bacterial strains

Nutrient media and cultivation methods

2. Analytical methods

Study of cell growth

Determination of enzyme activity

Quantitative determination of protein according to Bradford

Determination of pH and temperature optimum of enzymes

Study of the effect of ions and EDTA on enzyme activity

Quantitative determination of the obtained metabolites

Liquid chromatography with mass spectrometry (LC/MS)

3. Molecular biological methods

Isolation of total DNA from yogurt

Isolation of chromosomal DNA from L. bulgaricus

Isolation of plasmid DNA

Determination of the quality and quantity of the isolated NCs

Sequencing and bioinformatics analysis

Cloning of lacZ gene from L. bulgaricus 43 into vector pET41

Extraction of enzyme β-galactosidase

Purification of β-galactosidase from L. bulgaricus 43

Synthesis of GOS in vitro

Electrophoretic analysis of DNA and protein molecules

III. RESULTS AND DISCUSSION

1. Bacterial communities in homemade Bulgarian yogurts – metagenomic analysis

The remarkable properties of Bulgarian yogurt are primarily due to its unique microbial content. The microbial flora of yogurt is not a random collection of species, but a unique microecological niche [156]. The most studied yogurt starters rely on the symbiosis between *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* [157,158]. According to the definition of the Codex Alimentarius of the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), yogurt must contain no less than 107 viable colony-forming units (CFU) per gram of product. The starters produce volatile substances that are responsible for the typical flavor of yogurt (acetaldehyde, diacetyl, acetoin, acetone, ethanol, and 2-butanone), organic acids, and prebiotics such as galactooligosaccharides (GOS) [159].

Bulgarian yogurt is a symbol of our country and one of the most popular types of yogurt worldwide. The present study adds new, comprehensive information on the microflora of Bulgarian yogurt, in addition to the discovery of L. delbrueckii subsp. bulgaricus 120 years ago [25].

A collection of homemade Bulgarian yogurts from remote locations reveals a unique content of bacterial microflora. Given the significant influence of both starter cultures and accompanying LAB strains on the beneficial properties of yogurt [160], the present work aims to unveil the biodiversity of Bulgarian yogurts using metagenomics, isolate and identify new starter and accompanying LAB strains using modern genetic approaches, and investigate bioactive metabolites using precise analytical methods.

Several regions in Bulgaria are known to contain endemic microflora: the high mountains of Rila, Pirin and the Rhodopes, as

well as the Upper Thracian Plain. Fig. 1 indicates the geographical locations from which the homemade yogurt samples included in the metagenomic analysis were taken.

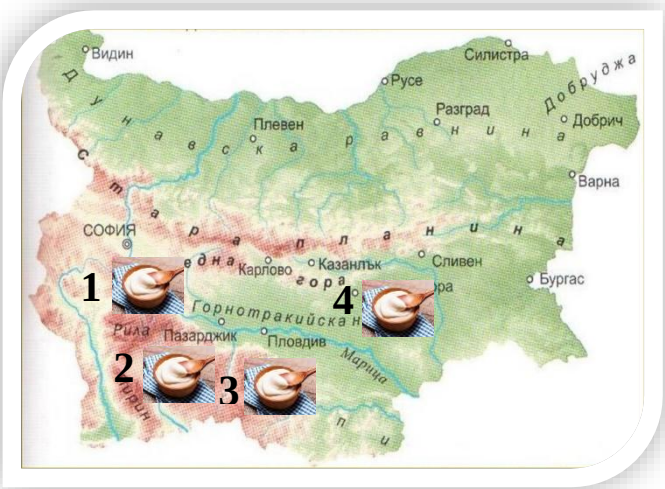

Figure 1. Geographical areas of homemade yogurt samples collected and subjected to metagenomic analysis

The preparation of the 16S metagenomic library from the samples and the sequencing itself were performed by Macrogen Inc. (Korea) using Herculase II Fusion DNA Polymerase and the Nextera XT Index Kit V2. The metagenomes were sequenced as 301-base fragments using Illumina technology, with Fast QC quality control. The assembly results showed that the quality-filtered data contained a total of between 73,209,822 and 82,104,372 bases and over 100,000 reads for each sample (Fig. 2). The percentage of quality reads with a quality score above Q20 and Q30 is shown in Fig. 2.

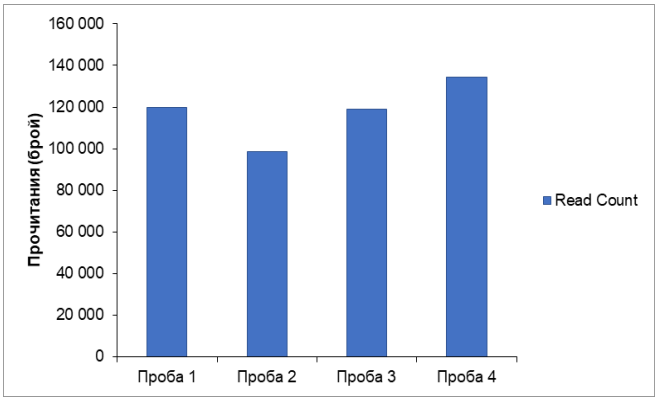

Figure 2. Number of reads in the sequencing analysis of the samples.

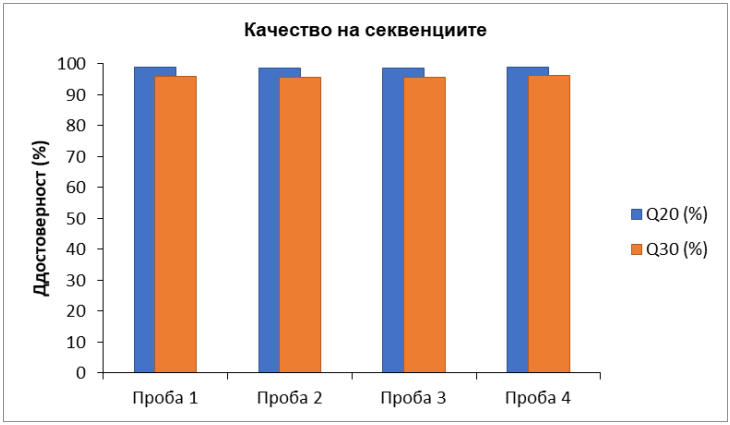

Figure 3. Quality control of metagenomic sequencing.

The raw sequencing data (SRA archive) were deposited in NCBI's GenBank with the following accession numbers: BioProject ID

PRJNA681359, BioSample SAMN16961356, and Sequence Read Archive (SRA) SUB8652830 (Fig. 4).

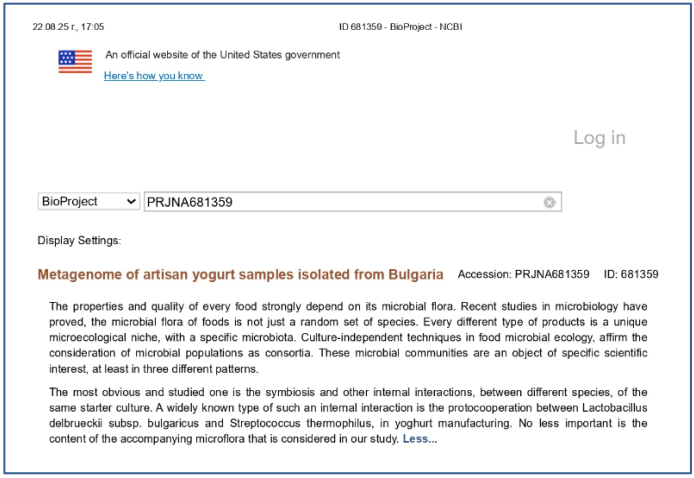

Figure 4. Deposited metagenomic sequences were published on the website of the National Center for Biotechnology Information (NCBI) of the USA.

Between 68.4% and 99.6% of the total lactic acid microbiota in the four studied samples consisted of two species: *L. delbrueckii* subsp. *bulgaricus* and *Str. thermophilus*, with the ratio between them varying widely. In Sample 2 (Rila), *Str. thermophilus* was the most abundant species, while in Sample 4 (Rhodopi), *L. bulgaricus* predominated.

Considering the regional distribution, the symbiosis between *L. bulgaricus* and *Str. thermophilus* exists in all samples, but the recommended ratio of ~1:1 between the strains is observed only in Sample 3 (from the Upper Thracian Lowland). This ratio in the starter significantly influences the qualities of the yogurt, including texture, color, flavor, and subsequent acidification.

Table 1. Metagenomic study of the abundance of lactic acid bacteria species in four samples of Bulgarian homemade yogurt prepared using own (homemade) starter. Origin of yogurt: Samples 1 and 2 - from villages in the Rila and Pirin mountains, Sample 3 - from the Upper Thracian lowland, Sample 4 - from the Rhodope Mountains.

	Relative abundance of the species in the sample (copy number)			
Species	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**
L. delbrueckii subsp. *bulgaricus*	22472	2500	15626	40010
Streptococcus thermophilus	10544	24249	18569	3603
Pediococcus acidilactici	314		649	-
Lactococcus lactis	98	4546	-	-
Lactococcus taiwanensis	-	3	-	-
Streptococcus saliviloxodontae	-	24	-	-
Leuconostoc lactis	-	116	-	-
Lactococcus garvieae	-	414	-	-
Lactobacillus equicursoris	78	0	208	175
Lacticaseibacillus rhamnosus	716	-	-	-
Lactobacillus helveticus	-	450	804	-
Limosilactobacillus fermentum	-	-	657	606
Lacticaseibacillus paracasei	-	395	-	524
Leuc. pseudomesenteroides	-	314	-	-
Leuc. mesenteroides	-	188	-	-

Rhodope yogurt is the richest source of *L. bulgaricus*, a species typical of Bulgarian yogurt that is less common in neighboring countries. For example, a Greek study reported the isolation of only one strain of L. delbrueckii subsp. bulgaricus from nine yogurts analyzed [161]. The relatively low number of L. bulgaricus in Sample 2 can be explained by the repeated transfer of the same starter culture to a non-sterile home environment. The explanation for this fact is provided by Urshev et al. (2006). Under both home and non-sterile conditions, bacteriophages can destroy part of the starter culture [162], thereby leading to an increase in the microbial number of the accompanying microflora [163].

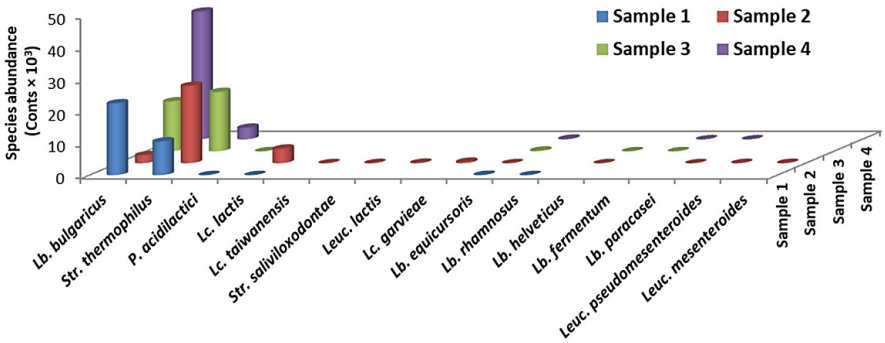

Figure 5. Metagenomic study of species abundance in four samples of Bulgarian yogurt prepared using homemade starter. Origin of yogurt: Samples 1 and 2, from villages in Rila and Pirin; Sample 3, from the Upper Thracian Lowland; Sample 4, from the Rhodope Mountains.

According to Sieuwerts et al. (2008), the traditional yogurt culture is composed of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*, and the name “yogurt” is only allowed for products containing starter cultures of these species [164]. Recent metagenomic studies comparing the microbiota of sour milk and the gut microbiome revealed the presence of *Lacticaseibacillus*

paracasei, *Lacticaseibacillus rhamnosus, L. parabuchneri, L. parafarraginis, Lactiplantibacillus plantarum, Lactococcus lactis, Leuconostoc mesenteroides,* and *Pediococcus acidilactici* in both niches [165,166]. Our results partially confirmed these observations, as *L. rhamnosus* and *L. paracasei* were found among the autochthonous lactobacilli in Bulgarian homemade yogurts. In addition, strains of *L. helveticus, Limosilactobacillus fermentum,* and *L. equicursoris* were also found (Table 1). However, while *L. helveticus* is frequently observed in Bulgarian yogurts and has been identified using genetic techniques [167,168], the presence of *L. fermentum, L. paracasei* and *L. rhamnosus* is unusual. *L. plantarum*, typical of Greek yogurt, was not detected in the samples studied. Although *L. equicursoris* belongs to the phylogenetic clade of L. delbrueckii, our study is the first to report its presence in yogurts.

This species was initially isolated from the feces of a thoroughbred racehorse by Morita et al. (2010) [169] and has recently been found in kefir [170]. Other associated ICD species are Lc. lactis, Lc. garvieae, Lc. taiwanensis, P. acidilactici, Leuc. lactis, Leuc. mesenteroides and Leuc. paramesenteroides. Some of these species are known to contribute to the sweet, fruity or vanilla aroma through the production of ethanol, 2-butanone, 2-pentanone and 2,3-pentanedione [171] and are currently preferred by consumers. Other desirable properties of these species are the synthesis of extracellular exopolysaccharides and high proteolytic activity.

Fig. 6 and Table 2 present the representatives of unwanted and even pathogenic bacterial microbiota detected by metagenomic analysis in homemade yogurt samples. Acinetobacter spp. and Chryseobacterium spp. were identified as the primary species responsible for the spoilage of yogurts. These Gram-negative bacteria, contaminants after the processing of fresh milk, severely limit the shelf life of the product. They overcome the microfiltration and subsequent pasteurization steps, and have also

been found in yogurts in Germany, Austria and Switzerland [172]. The presence of the Gram-negative, obligate anaerobe Bacteroides graminisolvens in two cow samples from different regions (samples 1 and 3) is indicative of soil contamination during milking and/or transportation of raw milk. This species is ubiquitous in barns and rural areas.

Table 2. Metagenomic study of the abundance of contaminant species in four samples of Bulgarian homemade yogurt. Origin of yogurt: samples 1 and 2, from villages in Rila and Pirin, sample 3, from the Upper Thracian Lowland, sample 4, from the Rhodope Mountains.

	Relative abundance of the species in the sample (copy number)			
Species	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**
Acinetobacter johnsonii	7	6	-	**4**
Citrobacter europaeus	7	3	-	**-**
Acinetobacter guillouiae	6	1	-	**1**
Acinetobacter ursingii	4	-	-	**-**
Moraxella osloensis	9	9	-	**6**
Pseudomonas japonica	2	-	-	**-**
Alcaligenes aquatilis	9	-	-	**3**
Bacteroides graminisolvens	12	-	1	**-**
Serratia nematodiphila	3	-	-	**-**
Acinetobacter beijerinckii	4	-	-	**-**
Chryseobacterium hominis	3	-	-	**-**

Stenotrophomonas nitritireducens	1	-	-	-
Comamonas testosteroni	1	-	-	-
Rummeliibacillus suwonensis	1	-	-	-
Acidovorax facilis	1	-	-	-
Aeromonas salmonicida	-	4	-	-
Str. ruminantium	-	**3**	**0**	-

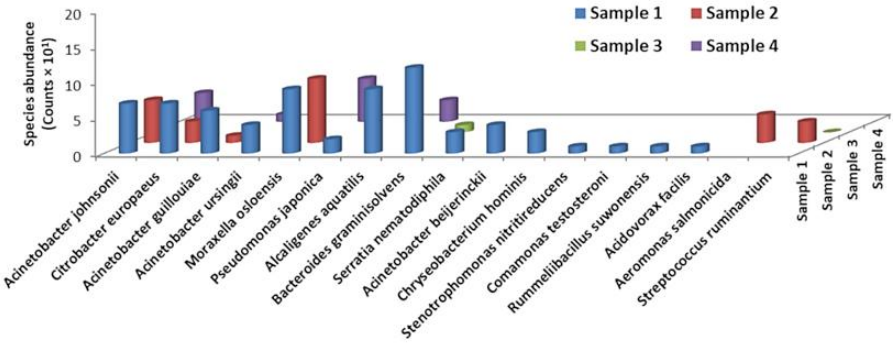

Figure 6. Metagenomic study of the species abundance of contaminating microflora in four samples of Bulgarian homemade yogurt. Origin of yogurt: Samples 1 and 2, from villages in Rila and Pirin; Sample 3, from the Upper Thracian Lowland; Sample 4, from the Rhodope Mountains.

Other microbial species, such as *Moraxella osloensis*, *Alcaligenes aquatilis*, *Str. ruminantium* and *Str. saliviloxodontae*, were found at levels between 0.1% and 0.01% or less. The low number of contaminating species (Table 2) suggests that single cells from the environment contaminated the milk; however, these species did not multiply due to the antagonistic activity of LAB [173].

Our observations align with studies by other authors, which report the presence of Gram-negative psychrotrophic contaminants in yogurts, such as *Pseudomonas* sp. and *Klebsiella* sp. [174]. Similarly, Bulgarian yogurts were contaminated, but with insignificant amounts of *Ps. japonica* and *Citrobacter europaeus*. However, dangerous zoonotic pathogens, such as coliform bacteria, *Salmonella* sp., *Listeria monocytogenes*, *Campylobacter jejuni*, *Yersinia enterocolitica*, *Brucella* sp., *Mycobacterium bovis,* or *Enterobacter sakazakii*, were not detected in any of the samples. The presence of these species in fresh milk has been previously reported by other researchers [175-177], but they are not present in yogurt.

Modern culture-independent techniques, such as metagenomics, enable the comprehensive study of yogurt consortia, revealing that they contain a diverse range of commensal microflora with beneficial properties [166].

These microbial communities are the subject of increasing scientific interest, in at least three different directions:

(1) Studying symbiosis and other internal interactions among lactic acid bacteria (LACBs) to select better quality starter cultures;

(2) Elucidating the specific dietary properties of yogurt;

(3) Identifying specific compounds produced by lactic acid bacteria (LACBs) that may address health problems without the use of drugs.

A study in healthy volunteers revealed that daily consumption of yogurt enriched with L. paracasei (L. casei 431®) improved the immune response by increasing NK cell activity and increasing interleukin (IL)-12 and immunoglobulin (Ig) G1 levels, as well as IFN-γ concentration [172]. Another strain of this species, L. paracasei CNCM I-1518, reduced bacterial translocation, intestinal dysbiosis, and ileal oxidative damage, and increased ileal β-defensin-1 expression in rats, suggesting an improvement in intestinal barrier integrity [179].

Yogurt containing *L. helveticus* H9 exhibited excellent ACE-inhibitory (ACE I) activity, characterized by high levels of antihypertensive peptides, such as Val-Pro-Pro and Ile-Pro-Pro [180,181]. L. helveticus LH-B02 is known to promote the formation of bioactive peptides such as αS1-CN f(24–32) and β-CN f(193–209) [182].

The patented *L. rhamnosus* GG (ATCC 53103) is the most studied probiotic strain in the world with more than 800 scientific studies. The authors claim that the strain tolerates the presence of bile salts, has a high affinity for human intestinal mucosal cells, and contributes to various health benefits, including the relief of gastroenteritis, diarrhea, eczema, and other diseases [183-185].

2. Gene sequencing and molecular structure of β-galactosidase from L. bulgaricus strain 43

In previous studies by the team, several *L. bulgaricus* strains with β-galactosidase activity were identified. One of them, *L. bulgaricus* strain 43, exhibited unusually high transgalactosidase activity, with synthesized amounts of GGT in lactose medium and in yogurt reaching 2.72 g/L and 2.83 g/L GGT after 48 h, respectively [25].

Therefore, the aim of this dissertation was the sequencing of the *lacZ* gene responsible for this function, its heterologous expression, and the molecular characterization of the β-galactosidase enzyme.

The gene was sequenced after obtaining a PCR product with a pair of primers amplifying *lacZ* (based on *lacZ* of the reference strains L. delbrueckii subsp. bulgaricus DSM 20080 and ATCC 11842). The resulting DNA fragment is 3027 bp in size and its sequence was deposited in NCBI GenBank with accession number OP617280..

```
LB43   121   IPSKNPLASYVRYFDLDEAFPEQTRLLLEDLIRTANVDRQNGHFVGYGEDSFTPSEFMVT  180
             IPSKNPLASYVRYFDLDEAF ++   L  D   TA     NGHFVGYGEDSFTPSEFMVT
L3     121   IPSKNPLASYVRYFDLDEAFWDKEVSLKFDGAATAIYVWLNGHFVGYGEDSFTPSEFMVT  180

LB43   181   KFLKKENNRLGVALYKYSSASWLEDQDFWRMSGLSGSVNLQAKPRLHLEDLKLTASLTDN  240
             KFLKKENNRL VALYKYSSASWLEDQDFWRMSGL  SV LQAKPRLHLEDLKLTASLTDN
L3     181   KFLKKENNRLAVALYKYSSASWLEDQDFWRMSGLFRSVTLQAKPRLHLEDLKLTASLTDN  240
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Figure 7. Comparison of the amino acid sequence of the region of lowest homology with other *L. bulgaricus* β-galactosidases. The comparison presented is based on the sequence of a well-studied β-galactosidase from *L. bulgaricus* strain L3 [148] as listed in NCBI GenBank (ACE06986).

The nucleotide sequence comparison showed 98.32% homology with lacZ of the reference L. delbrueckii ssp. bulgaricus ATCC 11842, and the corresponding protein sequence contained 97.52% identical amino acids with those of the other β-galactosidases found in this species. The lowest identity was observed in the sequences of regions 140–160 and 214–220 (Fig. 7), where 21 amino acid substitutions were identified compared to the characterized enzyme of *L. bulgaricus* strain L3.

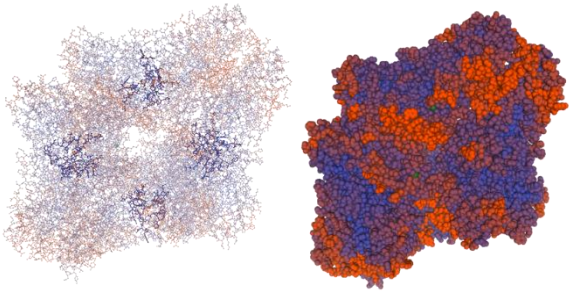


Figure 8. 3D model of β-galactosidase from *L. bulgaricus* strain 43, made by SWISS-MODEL Workspace. (a) Ball-and-stick representation of the chains revealing the formation of four active sites around the substrate; (b) Surface model of the tetramer.

Analysis of the protein sequence of the β-galactosidase molecule by SWISS-MODEL revealed that the enzyme is a homo-tetramer (Fig. 8). Each of the four chains contains 1008 amino acids and has a calculated molecular weight of 114.17 kDa.

Within 4 Å, twelve amino acid residues are essential for the formation of the active site and substrate binding: Asp207, His386, Asn464, Glu465, Met509, Tyr510, Glu532, His535, Tri562, Phen590, Asn593 and Tri980. The catalytic site is formed by Glu465 and Glu532 (which act as nucleophilic and acid-base catalysts) and Tyr510, which donates a proton to Glu532 to attack the substrate. Residues Tri562, Phen590, and Tri980 most likely act as analogues of Tri570, Phen616, and Tri593 in the β-galactosidase of Bacillus circulans ATCC 31382, i.e., they form the aromatic pocket that determines the type of bonds and the size of the product.

Two histidine residues (His386, His535), Asp207 and Tyr510 form hydrogen bonds with the substrate as part of the active site (Fig. 9).

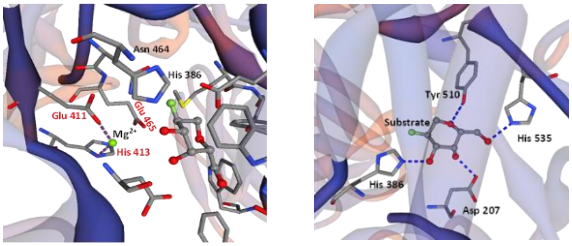


Figure 9. SWISS-MODEL Workspace/GMQE prediction of amino acid interactions in the active site of β-galactosidase from L. bulgaricus strain 43. (a) According to the model, Glu411, Glu465 and His413 bind to metal cations; (b) two histidine residues (His386, His535), Asp207 and Tyr510 form hydrogen bonds with the substrate.

3. Cloning, heterologous expression in *E. coli* BL21(DE3) and purification of β-galactosidase from L. bulgaricus 43

The β-gal (*lacZ*) gene from *L. bulgaricus* 43 (3024 bp, without the stop codon) was amplified by PCR with primers with ends containing sites for the corresponding restriction enzymes. The resulting fragment was cloned between the XhoI and NdeI sites of the vector pET-41b(+), replacing the gst gene to obtain the recombinant construct of 8044 bp in size (Fig. 10).

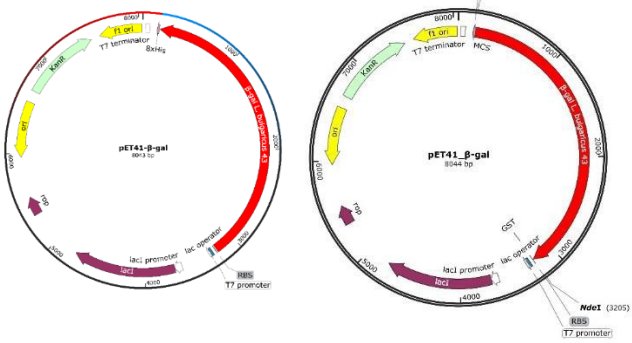


Figure 10. Physical maps of: a) the starting vector pET-41b(+); and b) the recombinant construct pET41_β-gal, based on a vector and containing the β-galactosidase gene of *L. bulgaricus* strain 43

The gene was placed under the control of a T7 inducible promoter and – due to the purposeful lack of a stop codon – fused to a His-tag sequence encoding a “tail” of 8 histidine residues. After transformation of competent E. coli DH5α cells (Fig. 11), the appropriate clones were confirmed by restriction analysis of the isolated plasmids as well as by sequencing**.**

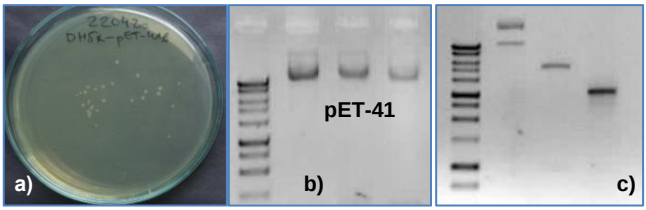


Figure 11. Transformation of E. coli DH5α: a) transformants with the vector pET-41(b); b) native vector after isolation from cells; c) vector and gene: purified fragments ready for ligation (native vector at the first start after the reference marker). Molecular marker: Perfect Plus Ladder (EURx).

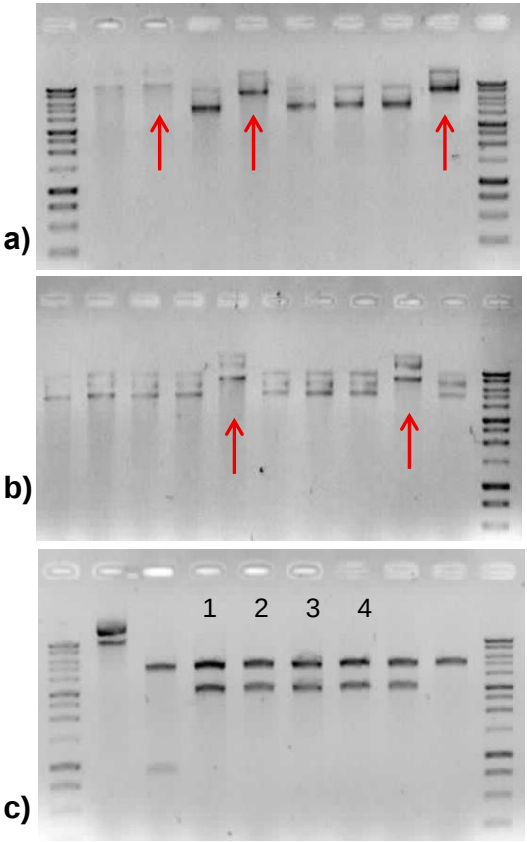


Figure 12. Analysis of clones in *E. coli* DH5α. a) and b) plasmids isolated from transformants; clones containing the β-gal gene are marked with an arrow; c) the identical five clones after restriction with XhoI and NdeI; for comparison: empty vector native and cut (left) and empty vector (without gene, right). Marker: Perfect Plus Ladder (EURx).

Table 3 shows the concentrations and purity of the recombinant pET41_β-gal constructs used for the transformation of *E. coli* BL21(DE3) by electroporation.

Table 3. Measured concentrations of plasmid DNA isolated from recombinant clones containing the pET41_β-gal construct.

Clone №	A260	A280	260/280	ng/ml
1	0.933	0.549	1.7	46.7
2	0.872	0.515	1.69	43.6
3	0.99	0.583	1.7	49.5
4	0.888	0.544	1.63	44.4
5	0.92	0.532	1.73	46.1

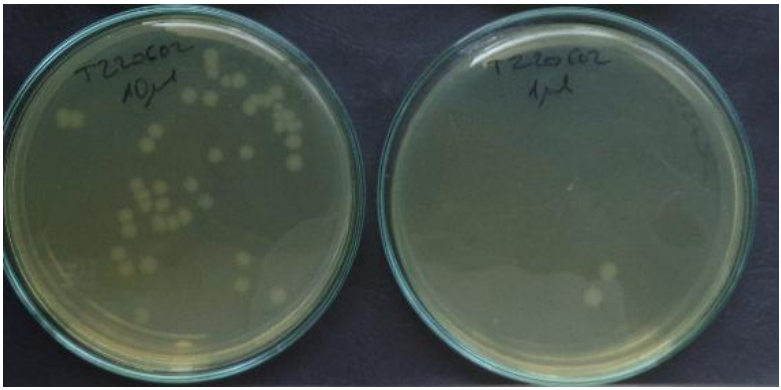


Figure 13. *E. coli* strain BL21(DE3) transformants containing the pET41_β-gal construct. Electroporation conditions: BioRad electroporator, Ec1 program 1.8 kV/mm, 5.7 ms

Overexpression of the recombinant protein in *E. coli* BL21(DE3) was achieved by induction of the T7 promoter with one mM IPTG for 24 h after reaching an OD600 of about 0.8. After a 1200-fold dilution, cell-free lysates were tested for β-galactosidase activity in potassium phosphate buffer (pH 7.2) at 37 °C with ONPG as the substrate. The maximum β-galactosidase activity reached 3015 ± 28 U/mL, corresponding to a specific activity of 2011 ± 16 U/mg.

Further purification of the enzyme was performed by affinity chromatography with gravity columns containing Ni-Sepharose (His Gravi Trap™). Optimization of elution with different imidazole concentrations, ranging from 100 to 1000 mM, revealed that the most effective concentration was 150 mM imidazole (Fig. 14).

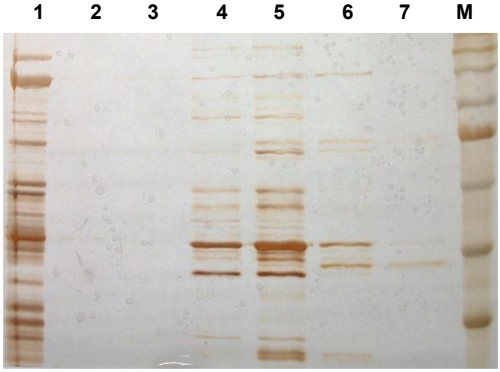


Figure 14. Purification steps of recombinant β-galactosidase by affinity chromatography with His Gravi Trap™ nickel-sepharose columns (GE Healthcare). Molecular marker: PerfectTM Tricolor Protein Ladder. Start number and sample: 1) cell extract, 2), 3) all proteins lost from the eluate, 4) elution at 200 mM imidazole, 5) 300 mM imidazole, 6) 150 mM imidazole, 7) 100 mM imidazole.

Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) showed the presence of a protein with the expected

molecular mass of approximately 115 kDa (Fig. 15), which was absent in the control cells – untransformed E. coli BL21(DE3). However, the purification of the target protein was accompanied by significant losses during elution, as well as by the presence of unwanted proteins. Therefore, some of the following experiments were performed with the unpurified enzyme.

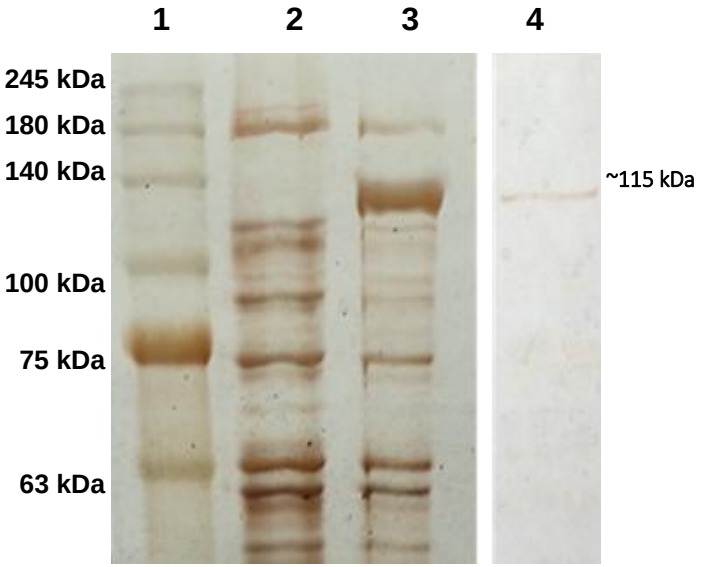


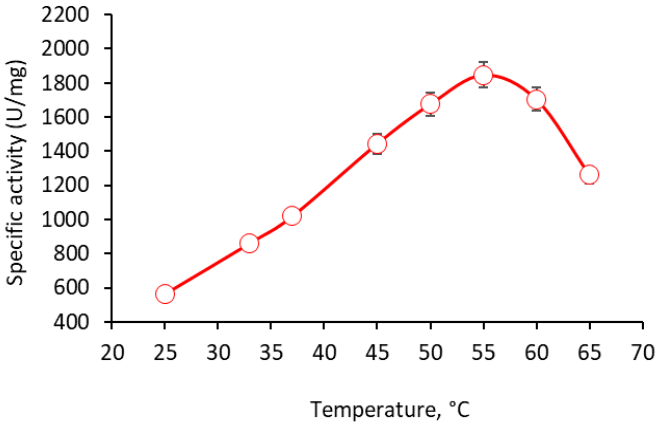
Figure 15. Overexpression and purification of recombinant β-galactosidase from L. bulgaricus 43 in E. coli BL21(DE3), demonstrated by SDS-PAGE in a 10% separating gel, after silver staining. Legend: (1) PerfectTM Tricolor Protein Ladder; (2) extract from untransformed E. coli BL21(DE3) cells as control; (3) extract from E. coli BL21(DE3) cells containing the pET-41_β-gal construct and induced with 1 mM IPTG for 24 hours, (4) purified β-galactosidase enzyme.

4. Biochemical characteristics of recombinant β-galactosidase

4.1. Effect of temperature and pH on enzyme activity

The recombinant β-galactosidase showed optimal hydrolase activity at 55°C (Fig. 16, a). In the range from 45 to 65 °C, no more than 25% loss of activity was observed. At 37 °C, the optimal activity was almost half (51.7%). The enzyme exhibits relatively high thermostability, as approximately 20% of its activity is retained after 24 hours at 55°C and about 15% after 60 minutes at 60°C.

Regarding pH, the recombinant enzyme showed optimal activity at pH 6.5 (Fig. 16, b). A loss of activity of less than 25% was observed over the relatively wide pH range from 5.5 to 7.5. The activity drops sharply at pH 5 and pH 8, reaching only 61% and 46% of the optimal values, respectively.



a)

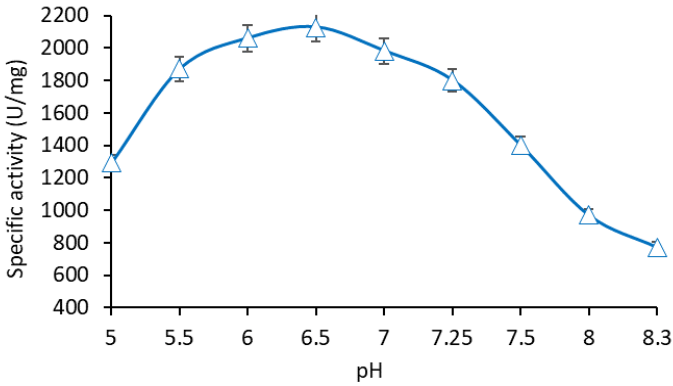


Figure 16. The influence of temperature and pH on the enzymatic activity of recombinant β-galactosidase from *L. bulgaricus* 43.

4.2. Effect of cations on the enzymatic activity of recombinant β-galactosidase

Eight different cations, ammonium, monovalent and divalent metal ions, were investigated for their possible influence on the hydrolytic activity of recombinant β-galactosidase with ONPG as substrate (Fig. 17). Ammonium ion $(NH_4)^+$, K^+, and Na^+ (as 10 mM salts) did not affect the enzyme. At the same time, the same concentrations of Cu^{2+} and Zn^{2+} weakly inhibited it. Mn^{2+}, $Mg^{2+,}$ and Ca^{2+} were chosen for further investigation, as they acted as enzyme activators. Under optimal conditions, manganese ions caused the most substantial increase in enzymatic activity, more than 3.5 times (compared to the control). At the same time, samples with magnesium and calcium showed almost twice the activity (90% and 98%, respectively) (Fig. 17).

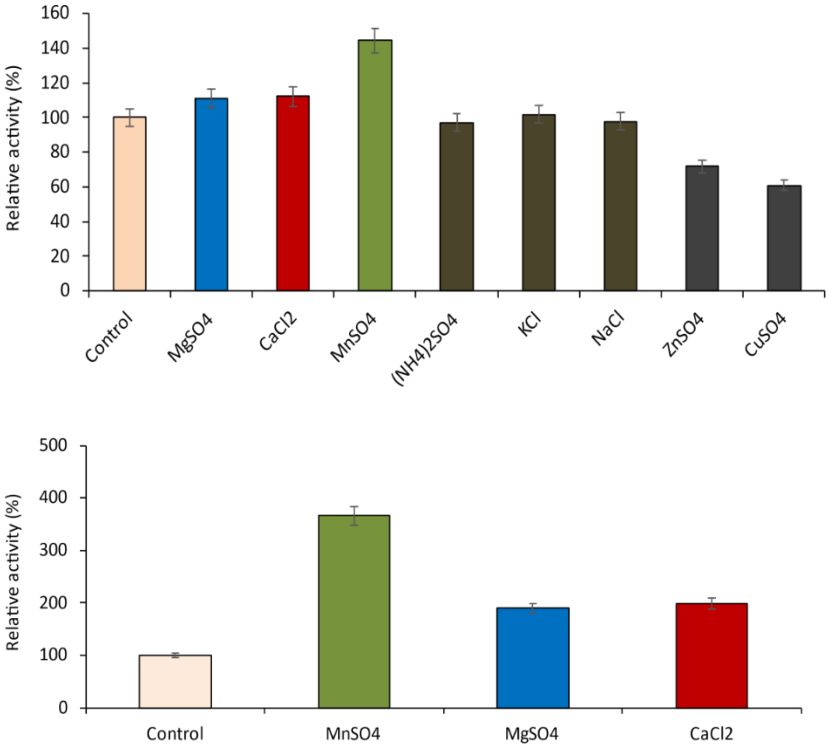


Figure 17. Effect of different cations on the activity of recombinant β-galactosidase at 10 mM salts; (a) assay conditions: 37 °C and pH 7.0; (b) assay conditions: 55 °C and pH 6.5.

4.3. Structure of the Galactooligosaccharides (GOS) Produced

Mass spectral qualitative analysis of GOS produced by L. bulgaricus β-galactosidase 43 from 40 g/L lactose revealed that the enzyme predominantly produced GOS with a degree of polymerization (DP) of 3.

Two types of DP3 molecules were found in approximately equal amounts. These structural isomers contain a galactose residue linked to lactose by β-(1→3) or β-(1→4) linkages. HRAMS analysis (as well as HPLC results for the heterologously expressed enzyme) showed that GOS with DP4 were produced in negligible amounts.

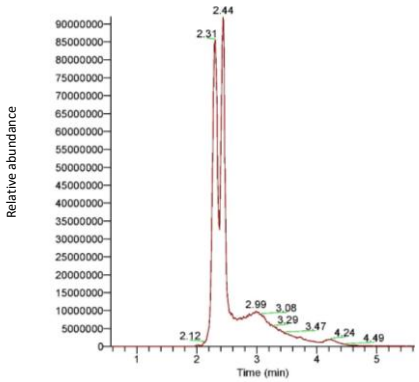


Figure 18. Extracted ion chromatogram for DP3 galactooligosaccharides (trisaccharides) obtained by β-galactosidase from L. bulgaricus 43. The peak at 2.31 min corresponds to a GOS with a β-(1→3) bond, while the peak at 2.44 min corresponds to a GOS with a β-(1→4) bond.

5. *In vitro* production of GOS by recombinant β-galactosidase

To determine the potential of recombinant β-galactosidase to produce GOS, different concentrations of lactose were incubated with 40 U/ml crude enzyme, and the products of the enzymatic reaction were analyzed by HPLC. The ability of recombinant β-galactosidase to synthesize GOS in vitro was significant and directly dependent on the substrate concentration (Fig. 19).

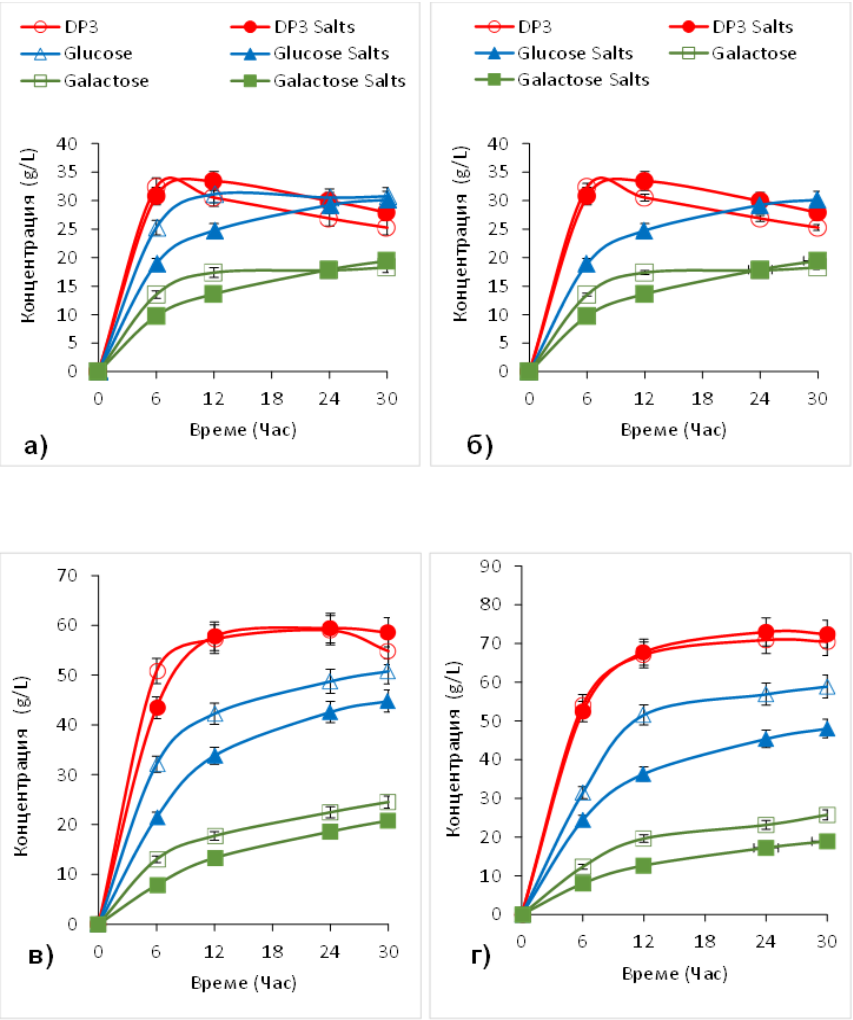


Figure 19. Time profiles of product formation from the action of recombinant β-galactosidase with different concentrations of lactose: (a) 80 g/L; (b) 120 g/L; (c) 160 g/L; (d) 200 g/L lactose. Legend: circles, DP3; triangles, glucose; squares, galactose. Open symbols, lactose + 1 mM MgSO4; closed symbols, lactose + 10 mM $MgSO_4$, 10 mM $MnSO_4$ and 10 mM $CaCl_2$.

GOS production reached peak values of 70.91 g/l GOS with DP3 after 24 h of incubation with an initial lactose concentration of 200 g/l (Fig. 19, d). This value was not only significantly higher than the values at the same time for 160 and 120 g/l lactose (20 and 67%, respectively), but also remained stable over the next six hours. Lower substrate concentrations increased the rate of the enzymatic reaction; at 120 and 80 g/l lactose, the peak of DP3 GOZ was reached after 12 or even only 6 hours, respectively.

The effects of metal cations on the transgalactosidase activity of recombinant β-galactose were radically different from those observed on their hydrolytic activity. The positive combined effect of 10 mM Mn^{2+}, Mg^{2+} and Ca^{2+} on GOS production was most pronounced at 80 g/l lactose, where an increase in DP3 GOZ synthesis was observed (about 10%). However, at higher lactose concentrations (120–200 g/l), this effect practically disappears. Concentrations of Mn^{2+}, $Mg^{2+,}$ and Ca^{2+} higher than 10 mM showed an inhibitory effect on DP3 GOS production both individually and in combination.

Galactooligosaccharides (GOS) are among the rare carbohydrates that fully meet the requirements for a prebiotic [120]. They are not hydrolyzed in the upper human gastrointestinal tract [186], cause a significant reduction in the number of harmful bacteria, enhance the growth of bifidobacteria in the colon [187,188], and cause a pronounced beneficial effect on the health of the consumer [189]. In addition to improving intestinal health [190], consumption of GOS favors calcium absorption and bone mineralization [191,192], alleviates lactose intolerance and prevents constipation [193], alleviates atopic dermatitis [194], regulates lipid metabolism, prevents obesity [195,196] and reduces the risk of colorectal cancer [197].

Prebiotic GOS include nondigestible oligomers containing β-linked galactose residues, with the exception of the disaccharides lactose and melibiose [121,122]. They vary in both

chain length and the manner in which the monomer units are linked, typically containing a terminal glucose and 2 to 8 galactose residues. In nature, GOS are present in small amounts in cow, camel, and human milk, and in large quantities in kangaroo milk [198]. Due to the high demand for infant formulas and prebiotics, commercial production of GOS is increasing by 6% per year [186], and the global market is expected to reach US$10.55 billion by 2025 [154]. GOS are commercially produced by chemical methods, which are not preferred because they generate undesirable by-products [148], or by enzymatic synthesis from lactose by β-galactosidases of fungal or bacterial origin. The most commonly used enzymes are those of *Kluyveromyces lactis* and *Aspergillus oryzae*, producing mainly β-(1→6)-linked GOS, and those of *Bacillus circulans* for β-(1→4)-linked GOS. However, some probiotic strains grow more efficiently on GOS produced by their own β-galactosidases than on commercial GOS [154]. Furthermore, enzymes from lactobacilli and bifidobacteria have recently received special attention regarding their transgalactosylation activity, as they tend to catalyze this reaction predominantly [154, 199].

According to the CAZy database [200], β-galactosidases (EC 3.2.1.23) are structurally diverse enzymes. Based on their amino acid sequences, hydrophobic clusters, reaction mechanism, and conservation of catalytic residues, they are classified into the first, second, thirty-fifth (GH35), and forty-second (GH42) families of glycoside hydrolases.

GH2 family beta-galactosidases have been identified in various Lactobacillus species. These include the traditionally described species L. acidophilus, L. coryniformis, and L. johnsonii, as well as the new taxonomic groups Lactiplantibacillus plantarum, Limosilactobacillus reuteri, and Latilactobacillus sakei. They are encoded by the lacL and lacM genes and form heterodimers of the LacLM type [200-202]. In contrast, the β-galactosidases of L. helveticus and *L. delbrueckii* subsp. *bulgaricus* are encoded by a

single gene (homomeric, LacZ type) and have been much less studied [123,203-206]. In addition to the fact that the available data on β-galactosidases of *L. bulgaricus* were quite scarce to date, they were also misleading. Until a decade ago, it was believed that L. bulgaricus strains usually synthesize a truncated, inactive enzyme [204]. Only with the accumulation of data from whole genome sequencing of L. bulgaricus has the complete sequence of the responsible genes become clear [213].

Our results show that the newly isolated L. bulgaricus strain 43 can spontaneously form large amounts of GOS with DP3 in yogurt. Furthermore, these GOS structures contain β-(1→4) linkages between galactose monomers, which is reported for the first time in GOS produced by this species.

The reasons for this unusual enzymatic activity are genetically determined. The *lacZ* gene of *L. bulgaricus* 43 encodes a novel enzyme that has 21 amino acid substitutions compared to known β-galactosidases from *L. bulgaricus*. These substitutions are essential for the enzyme's properties, as they are located in proximity to the active site at the N-terminus. A 3D model of the molecule revealed that the enzyme from strain 43 functions as a tetramer, which has not been reported for L. bulgaricus and is most likely due to the multiple amino acid substitutions in the 140–220 region. According to Weber and Schneider [207], protein dimers are stabilized by areas rich in amino acids with small side chains such as glycine, alanine, or serine, many of which contain Gx-like motifs. In the sequence of β-gal of *L. bulgaricus* 43, 3 new such amino acid residues appear – two glycines at positions 191 and 215 and one serine at position 214. Considering that in E. coli the active sites of the enzyme are formed by chain interactions and that the “SV”, “GV” and “SG” motifs stabilize the dimeric structures [208], these substitutions These are most likely the reason for the tetrameric structure of the enzyme in L. bulgaricus strain 43 and its high activity. The structure of the catalytic center of β-gal is consistent with the bioinformatic analysis by Bultema et al. [209],

who studied the structure of BgaD from *B. circulans*, a similar enzyme belonging to the same glycoside hydrolase family (GH2). Compared to BgaD, the *L. bulgaricus* 43 enzyme contains a conserved residue, Glu532, which serves as the nucleophile in the enzymatic reaction. The role of an acid/base catalyst in β-gal is played by Glu465 (Glu447 in B. circulans). Similarly, two histidine residues His386 and His535 are present in the active site. It is noteworthy that, like β-gal, B. circulans BgaD forms trisaccharides with β-(1→4)-linkages as the main transgalactoside product [210]. One difference, however, is that BgaD of B. circulans is a Mg2+-independent enzyme. In contrast, β-gal of *L. bulgaricus* 43 requires Mg^{2+} (at least 0.5 mM) for its activity, as this divalent metal cation acts as a cofactor in the catalytic center. The magnesium ion can often be replaced by Mn^{2+}, which our results confirm with a 366% increase in the enzymatic activity of β-gal in the presence of 10 mM MnSO4.

The influence of metal ions on the enzymatic activity is very different from that reported for other known β-galactosidases from *L. bulgaricus*. For example, Nguyen et al. [203] cloned and overexpressed the lacZ gene from *L. bulgaricus* DSM 80021 in *L. plantarum*. The resulting enzyme is activated by K+ and Na+ (more than 5- and 10-fold at 10 mM, respectively) and inhibited by Ca2+ and Mg2+ (more than 60% at 10 mM), in contrast to β-gal of strain 43. Similar metal sensitivity has been reported for β-galactosidases from other species, for example, the enzyme from *L. leichmannii* 313, which is also activated by Na^{+} (5-fold at 10 mM), inhibited by Ca^{2+} (almost 50%) and Mn^{2+} (nearly 70%, both at 10 mM), but is unaffected by K^{+} (Table 13).

Considering the other parameters, the pH optimum of the enzyme from *L. leichmannii* 313 is 5.5, and it is very narrowly limited: the authors report more than a 50% loss of activity within half a pH unit in either direction [217].

Again, the pH optimum is significantly different for our enzyme, which loses the same activity over a threefold wider range

of pH values. The temperature optimum of 55 °C for the enzyme of strain 43 enables the production of GOP at elevated temperatures, thereby increasing the solubility of lactose and enhancing the process productivity.

Table 13. Enzyme activity, GOP production, and influence of metal ions on the specific enzyme activity of selected microbial β-galactosidases. CEA = specific enzyme activity. Substrates: O = ONPG; L = lactose.

Species, Strain	Type of Enzyme [1]	Lactose (g/L)	GOS (g/L)	GOS (%) [2]	SEA [3] (U/mg)	Metal Ions [4]
L. bulgaricus 43	Crude	200	70.91 (DP3)	34 (DP3)	2011 (O)	↑ Mn^{2+}, Mg^{2+}, Ca^{2+} ↓ Zn^{2+}, Cu^{2+}
L. bulgaricus CRL450	Cell-free extract	300	n/a	41.3	2.06 (O)	n/a
L. bulgaricus DSM 20081	Purified, non-His-tag	205	102	50	317 (O) 123 (L)	↑ Na^{+}, K^{+} ↓ Mg^{2+}, Ca^{2+}
L. bulgaricus wch9901	Crude	n/a	n/a	n/a	6.2 (O)	n/a
L. acidophilus R22	Purified natural	205	n/a	38.5	361 (O) 28.8 (L)	↑ Mg^{2+} ↓ Mn^{2+}, Cu^{2+}, Zn^{2+}
Lim. fermentum K4	Purified	200-400	n/a	37	184 (O) 41 (L)	↑ Na^{+}, K^{+}, Mg^{2+}
L. helveticus DSM 20075	Purified	205	n/a	n/a	476 (O) 11.1 (L)	↑ K^{+}, Na^{+} ↓ Mn^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}

Species, Strain	Type of Enzyme [1]	Lactose (g/L)	GOS (g/L)	GOS (%) [2]	SEA [3] (U/mg)	Metal Ions [4]
L. leichmannii 313	Purified	n/a	n/a	n/a	31.28 (O)	↑ Na^{+} ↓ Ca^{2+}, Mn^{2+}
Bifidobacterium breve DSM 20213	Purified (2 enzymes)	200	n/a	33-44	489 (O) 59 (L)	n/a
Bif. longum *Bif. pseudocatenulatum*	Purified (2 enzymes)	n/a	n/a	n/a	2200 (O) 0.58 (O)	↑ Zn^{2+}, Na^{+}, Ca^{2+}, Mn^{2+} ↓ Al^{3+}
B. circulans	commercial	400	198	41	n/a	n/a
Pyrococcus woesei	Purified	n/a	n/a	n/a	5400 (O)	n/a
Aspergillus oryzae	commercial	400	107	26.8	n/a	n/a

[1] All recombinant and His-tagged unless otherwise noted; [2] Percentage of total sugars; [3] SEA, specific enzyme activity (U/mg protein); substrates: O = ONPG; L = lactose; [4] Activators/inhibitors of SEA; ↑, activation; ↓, inhibition; 1U = µmol/min.

Since the enzymatic activity of β-galactosidase in *L. bulgaricus* is generally low [30], heterologous expression of the corresponding gene in different microbial hosts is the preferred method for studying the enzyme and for its application in synthesizing prebiotic GOS [203]. However, the highest activities of recombinant enzymes reported so far are just over 300 U/mg (Table 13), more than six times weaker than the β-galactosidase of *L. bulgaricus* 43. Recombinant enzymes obtained from other Lactobacillus spp., such as *L. helveticus* and *Limosilactobacillus fermentum*, have specific activities of nearly 500 U/mg. Many of these studies report significant substrate-related differences, with activity with ONPG being 3 to 40 times higher than with lactose.

This discrepancy should be considered when evaluating potential industrial applications of the enzyme. The same applies to claims that recombinant enzymes with histidine tags typically show 20-30% lower activity [203]. Regarding GOS yields, *L. bulgaricus* strains have an advantage over other lactobacilli, although even within these strains, half of the total sugar content remains undigested. The present study exemplifies the successful conversion of lactose to a substantial amount of GOS. The unique sensitivity of strain 43's β-galactosidase to metal cations offers opportunities for optimization that warrant further investigation.

The development of functional foods is one of the most important and rapidly advancing directions in modern science and practice, combining knowledge from fields such as microbiology, nutrigenomics, biotechnology, and food chemistry. Functional foods are defined as products that, in addition to their traditional nutritional value, have a proven beneficial effect on the body's physiological functions and help prevent various diseases. In this context, studying traditional Bulgarian fermented milk products is particularly important because they are a unique source of naturally occurring and rich microbiota, accumulated and preserved over centuries. This microbiota not only gives the products their characteristic taste and aroma but also possesses biological activity, which remains the focus of ongoing scientific research. Among all Bulgarian dairy products, yogurt holds a central place—both in national cultural identity and scientific interest. Although its microbiological basis was described over 120 years ago, new aspects of its importance for human health continue to be uncovered. The symbiosis between the two main species in starter cultures—*L. bulgaricus* and *Str. thermophilus*—remains a Bulgarian tradition, but the accompanying microorganisms in local and regional yogurt variants reveal an endless diversity of biodiversity, with the potential yet to be systematically studied. This dissertation specifically contributes to this field by providing new

data on the composition, enzyme profile, and metabolic capabilities of strains originating from authentic Bulgarian products.

This work has led to the discovery of new aspects of the genetic and functional diversity of Lactobacillus bulgaricus – a microorganism that has long transcended national borders and has transformed the Bulgarian scientific and culinary tradition into a globally recognized one. In conclusion, the scientific novelties of the dissertation work can be systematized as follows: (1) for the first time, specific strain differences in the enzyme profile of L. bulgaricus have been described, which determine different potential for the synthesis of functional oligosaccharides; (2) data on the accompanying microflora of homemade yogurts from different regions of Bulgaria are provided, which highlights the richness and uniqueness of the national microbial heritage; (3) new perspectives for using the isolated microorganisms in the development of value-added foods aimed at strengthening intestinal health are demonstrated. The results obtained aim to enrich the fundamental knowledge in the field of ICD microbiology, but also to expand the practical applications of these strains in the food industry and healthcare. The analysis can be a basis for future scientific research, as well as for the development of new high-value-added products that preserve and build on the national tradition, while responding to global trends for healthy and sustainable nutrition.

V. CONCLUSIONS

1. The metagenomic analysis confirmed that L. bulgaricus and S. thermophilus are dominant in homemade yogurts, but in different regions of Bulgaria their ratio varies significantly.

2. In homemade yogurts, L. rhamnosus, L. paracasei, L. helveticus, L. fermentum were found as concomitant species of lactic acid bacteria.

3. Rare species of LAB were found, including L. equicursoris, withreported for the first time in yogurt.

4. The lacZ gene from L. bulgaricus strain 43 encodes a novel β-galactosidase enzyme with 21 unique amino acid substitutions, providing its higher activity compared to other similar enzymes in the species.

5. The amino acids Glycine 191, Glycine 215 and Serine 214 determine the tetrameric structure of the β-galactosidase of L. bulgaricus strain 43.

6. Successful heterologous expression of the enzyme in E. coli BL21 (DE3) led to the production of a recombinant enzyme with enormous activity (over 3000 U/ml).

7. The recombinant enzyme exhibits specific sensitivity to Mg^{2+}, Mn^{2+} and Ca^{2+} and reaches an activity over 2000 U/mg, significantly higher than all known to date.

8. Due to its potent transgalactosidase activity, the enzyme synthesizes up to 70.9 g/l of GOS (mainly DP3), which shows great potential for industrial application.

9. The β-Galactosidase of L. bulgaricus strain 43 forms atypical, β-(1→4) linkages between monomers in GOS, which contributes to an increased prebiotic effect of the spontaneously synthesized GOS in Bulgarian yogurt.

VI. CONTRIBUTIONS

1. For the first time, a metagenomic profile of domestic Bulgarian yogurts from different geographical regions is presented, which reveals both dominant and rare and new types of MCD.

2. The presence of L. equicursoris in yogurt is documented – a novelty for the scientific literature.

3. A strain of L. bulgaricus 43 with unusually high transgalactosidase activity is identified.

4. The lacZ gene of the strain was sequenced and characterized, showing 21 unique amino acid substitutions associated with a tetrameric structure and high enzymatic activity.

5. The successful heterologous expression of the recombinant β-galactosidase has led to a significant industrial potential of the enzyme for the production of GOJ with prebiotic properties.

PUBLICATIONS RELATED TO THE DISSERTATION

1. **Ivanov, I.**; Petrov, K.; Lozanov, V.; Hristov, I.; Wu, Z.; Liu, Z.; Petrova, P. Bioactive Compounds Produced by the Accompanying Microflora in Bulgarian Yoghurt. Processes 2021, vol. 9, 114. https://doi.org/10.3390/pr9010114 (Q2, cited 19 times in SCOPUS)
2. Petrova, P.; **Ivanov, I.;** Tsigoriyna, L.; Valcheva, N.; Vasileva, E.; Parvanova-Mancheva, T.; Arsov, A.; Petrov, K. Traditional Bulgarian Dairy Products: Ethnic Foods with Health Benefits. Microorganisms 2021, vol. 9, 480. https://doi.org/10.3390/microorganisms9030480 (Q2, cited 42 times in SCOPUS)
3. Arsov, A.; **Ivanov, I.;** Tsigoriyna, L.; Petrov, K.; Petrova, P. In Vitro Production of Galactooligosaccharides by a Novel β-Galactosidase of Lactobacillus bulgaricus. Int. J. Mol. Sci. 2022, vol. 23, 14308. https://doi.org/10.3390/ijms232214308 (Q1, cited 12 times in SCOPUS)

OTHER PUBLICATIONS:

4. Petrova, P.; Arsov, A.; **Ivanov, I.;** Tsigoriyna, L.; Petrov, K. New Exopolysaccharides Produced by Bacillus licheniformis 24 Display Substrate-Dependent Content and Antioxidant Activity. Microorganisms 2021, 9, 2127. https://doi.org/10.3390/microorganisms9102127 (Q2, cited 24 times in SCOPUS)

CONFERENCE PRESENTATIONS

1. Ivanov, I., Petrova P. "Prebiotics producing Lactobacillus bulgaricus strains isolated from Rhodope yogurts" 69th Scientific conference with international participation "Food science, technique and technologies - 2022", 29.09.2022 - 30.09.2022, Plovdiv, Bulgaria (Poster report)

2. Ivanov, I., Arsov., A., Petrova P., Petrov K. "New Beta-galactosidases from Lactic Acid Bacteria", XVth Congress of the Bulgarian Microbiologists with International Participation, 05.10.2022 - 08.10.2022, Koprivshtitsa, Bulgaria (Report)

3. Ivanov, I., Arsov, A., Tsigoriyna, L., Petrov K., Petrova P. "Novel β-Galactosidase of Lactobacillus bulgaricus: a molecular study of its dual enzyme activity", Annual Meeting of the NNP "Healthy Foods for a Strong Bioeconomy and Quality of Life", 12.06.2023 - 13.06.2023, Plovdiv, Bulgaria (Poster presentation).

4. Petrova P., Arsov A., Ivanov I., Tsigoriyna L., Valcheva N., Petrov K. Cloning and heterologous expression of exo-cellulase gene cbhA in Bacillus licheniformis for direct production of 2,3-butanediol from biomass. ORAL Presentation 3BO.14.2, Section "Biochemical Production of Alcohols and Chemicals", 29-th European Biomass Conference & Exhibition (EUBCE), 26 - 29 April, 2021, (Presentation).

5. Petrov K., Valcheva N., Tsigoriyna L., Arsov A., Ivanov I., Petrova P. Bacillus velezensis, Bacillus toyonensis, and Bacillus safensis are able to produce 2,3-butanediol from lignocellulosic sugars. Poster presentation 3AV.6.14, Section "Biochemical Production of Alcohols and Chemicals", 29th European Biomass Conference & Exhibition (EUBCE), 26 - 29 April 2021, (Poster presentation).

6. Arsov A., Petrova P., Ivanov I., Tsigoriyna L., Petrov K. Heterologous expression of cellulosome genes of Hungateiclostridium thermocellum in Bacillus strains. Poster presentation PP-13, 13-th International Conference on Protein Stabilization (PROTSTAB), 07 - 09 October, 2021, (Poster presentation).