СОФИЙСКИ УНИВЕРСИТЕТ
„СВ. КЛИМЕНТ ОХРИДСКИ“

БИОЛОГИЧЕСКИ ФАКУЛТЕТ

SOFIA UNIVERSITY
St. KLIMENT OHRIDSKI

FACULTY OF BIOLOGY

БИОЛОГИЧЕСКИ ФАКУЛТЕТ
СОФИЙСКИ УНИВЕРСИТЕТ

FACULTY OF BIOLOGY
SOFIA UNIVERSITY

FACULTY OF BIOLOGY

DEPARTMENT OF **BIOTECHNOLOGY**

Nikola Nikolov Atanasov

PROPERTIES AND CHARACTERISTICS OF NEWLY ISOLATED LACTIC ACID BACTERIA STRAINS AND APPLICATION IN MODEL PROBIOTIC PRODUCTS FOR ORAL HEALTH

A B S T R A C T

OF PhD THESIS

for awarding the educational and scientific degree **“Doctor”**
in the professional field **5.11. Biotechnologies**,
Technology of biologically active substances

Scientific supervisor: assoc. prof. Dilyana Nikolova

Sofia
2024

❖❖❖

I would like to thank my supervisor, assoc. prof. Dilyana Nikolova, for the collaborative work, guidance, recommendations and shared professional experience in the development of my thesis, as well as for creating a positive working environment.

I would like to thank assoc. prof. Yana Evstatieva and colleagues from the Department of Biotechnology for their cooperation and support during the work process, for sharing their experience and for maintaining positive collegial relations.

I would like to thank assist. prof. Slavil Pejkov for the professional consultation during the genetic and bioinformatics analyses.

I would like to thank my colleagues, Bachelor and Master of Biotechnology graduates, Petya Penkova, Martina Mihaylova-Varbeva and Denis Borisov, with whom we worked in collaboration and supported each other's research growth.

I would like to thank my family for their moral support.

❖❖❖

The dissertation contains a total of 149 pages in A4 format, 18 tables and 26 figures of which 15 tables and 21 figures are in Results and Discussion. The reference list includes 467 references, of which 1 is in Bulgarian and 466 are in English.

The dissertation research work was funded through:

1. Project: Targeted isolation and characterization of lactic acid bacteria strains from the resident microbiota in the human oral cavity or from specific food products and determination of basic probiotic properties, contract № 80-10-69/ 25.03.2021.
2. Project: Evaluation of probiotic and technological characteristics of newly isolated strains of oral lactic acid bacteria, contract № 80-10-78/11.05.2022.
3. National Scientific Programme "Healthy Foods for a Strong Bioeconomy and Quality of Life" 2018-2023, approved by Decision of the Council of Ministers No. 577/17.08.2018.

The dissertation is scheduled for defense by order of the Rector of Sofia University "St. Kliment Ohridski" ..... and the defense will take place on ..... at ..... before the Scientific Jury, composed of:

1. ..... ;
2. ..... ;
3. ..... ;
4. ..... ;
5. ..... .

Abbreviations

LAB – lactic acid bacteria
GRAS – generally recognised as safe
QPS – qualified presumption of safety
FAO – Food and Agriculture Organization of the United Nations
WHO – World Health Organization
FDA – U.S. Food and Drug Administration
EFSA – European Food Safety Authority
GIT – gastrointestinal tract
SML – lyoprotective medium w/ 10 % skimmed milk, 5 % lactose and 2 % ascorbic acid
SMT – lyoprotective medium w/ 10 % skimmed milk, 5 % trehalose and 2 % ascorbic acid
MRS – de Man-Rogosa-Sharpe broth/agar
MH – Mueller-Hinton agar
ME – Malt extract broth/agar
TYCSB – Tryptone Yeast Extract Cystine w/ Sucrose and w/o Bacitracin agar
BHI – Brain Heart Infusion broth/agar
PBS – phosphate buffered saline solution
BSA – bovine serum albumin
DNA – deoxyribonucleic acid
rRNA – ribosomal ribonucleic acid
PCR – polymerase chain reaction
EPSs – exopolysaccharides
ROS – reactive oxygen species
BLAST – Basic Local Alignment Search Tool
MALDI-TOF MS – matrix-assisted laser desorption ionizing time-of-flight mass spectrometer
PMF – peptide mass fingerprint
MAR – multiple antibiotic resistance
IgA – immunoglobulin A
NCBI –National Center for Biotechnology Information
CLSI – Clinical & Laboratory Standard Institute
SD – standard deviation
CFU – colony forming units

I. Introduction

In recent decades, there has been a significant increase in scientific activity focused on the study of probiotic microorganisms. A number of scientific studies have established that many probiotic strains of the lactic acid bacteria (LAB) group have beneficial effects in the prevention and therapy of oral infectious diseases. In the course of increasing research, the beneficial properties of probiotic strains to interact with the oral microbiota and maintain a healthy microbial balance have been established.

LAB include a wide variety of representatives that, through their long probiotic history, have become part of human life. They are incorporated into our daily life in the form of naturally occurring products, food products that have undergone fermentation processes with the help of starter cultures, and various food supplements with probiotic uses. LAB also naturally colonise the human body by contributing in various ways to its health status.

The oral microbiome plays an important role on the human microbial community as well as on human health. As the oral cavity is the starting point of the gastrointestinal tract (GIT), the development of oral diseases can also contribute to the occurrence of systemic diseases. Therefore, many research efforts are focused on investigating processes that inhibit pathogenic microorganisms and maintain oral health.

The level of oral hygiene is of utmost importance and has a significant impact on the composition and condition of the oral microbiome. Maintaining good oral health affects a person's ability to adapt to physiological changes throughout life and to maintain their dentition and oral cavity through independent self-care. Probiotics targeting the oral cavity can successfully compete with pathogenic microorganisms and increase the presence of beneficial bacteria, and can thus contribute positively, especially to the prevention of oral diseases, including dental caries, periodontal disease and halitosis, and to the therapy of their occurrence. Therefore, the study of LAB as natural antagonists of various pathogens as well as their beneficial functional properties continues to be of considerable interest to modern science. Also, research on their probiotic capabilities is essential for their incorporation into various dietary supplements in order to develop effective functional products.

II. Objective and tasks

LAB with probiotic potential can be beneficial for the prevention of different infections in the oral cavity. They also maintain healthy oral cavity through interactions with the oral microbiome providing a healthy microbial balance. Many positive results from worldwide scientific research, aiming at oral health, prove the development in this subject. Continuation of the scientific research will enrich this scientific topic with data with newly isolated LAB strains, determining their beneficial functionalities, as well as their application in new formulations for functional products for oral health.

The objective of the dissertation is:

Isolation, identification and characterisation of new LAB strains from the oral microbiome, evaluation of their probiotic potential, antimicrobial interations, and investigating their potential application in a model probiotic product for oral health.

Tasks have been formulated to achieve the set objective:

1. Isolation and characterization of LAB strains from the human oral cavity.
   1.1. Evaluation of morphological and physiological characteristics.
   1.2. Screening for enzymatic activities of the newly isolated LAB strains.
2. Species identification of the newly isolated LAB strains.
   2.1. 16S rDNA gene sequencing analysis.
   2.2. Protein profiling by the MALDI-TOF MS system.
   2.3. Bioinformatics whole genome sequencing and processing.
3. Evaluation of survival ability and growth dynamics of the tested LAB strains in GIT.
   3.1. Survival ability in simulated oral conditions.
   3.2. Survival ability under direct exposure to stress factors in GIT.
   3.3. Growth dynamics in the presence of GIT stress factors.
4. Evaluation of the adhesive properties of the tested LAB strains.
   4.1. Autoaggregation capability.
   4.2. Mucin binding capability.
   4.3. Biofilm formation ability.
   4.4. *In silico* bioinformatics analysis for presence of adhesion proteins.
5. Evaluation of antibiotic resistance and bioinformatics analysis for acquired genes of the tested LAB strains.
6. Antioxidant capacity screening of the tested LAB strains.
7. Antagonistic interactions of the tested LAB strains against test-pathogens, including oral test-pathogens.

7.1. Antimicrobial activity screening against test-pathogens and bioinformatics analysis for bacteriocin producing genes.
7.2. Antagonistic activity against oral test-pathogens by co-cultivation.
7.3. Co-aggregation capability.
7.4. *In vitro* evaluation of antibiofilm activity against test-pathogens.

8. Survival ability of the tested LAB strains after freeze-drying and storage.

9. Stability and survival ability of selected LAB strains in different model product formulations for oral health.
9.1. Probiotic potential calculation and selection of strains for inclusion in the composition of model probiotic products.
9.2. Application of the selected strains in different model products as fresh concentrated culture and viability evaluation.
9.3. Application of the selected strains in a model product in freeze dried state and viability evaluation.
9.4. Side microflora evaluation of the prepared model products.

III. Materials and methods

1. Test-microorganisms

Bacillus cereus ATCC 11778, *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231, *Escherichia coli* ATCC 25922, *Propionibacterium acnes* (isolate), *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* subsp. *aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Streptococcus mutans* ATCC 25175.

2. Growth media and solutions

Powdered or granulated growth media were used, prepared according to the manufacturer's instructions: de Man-Rogosa-Sharpe (MRS) broth/agar (Merck; HiMedia); Mueller-Hinton (MH) agar (Merck); Malt extract (ME) broth/agar (Oxoid); Tryptone Yeast Extract Cystine w/ Sucrose and w/o Bacitracin (TYCSB) agar (HiMedia); Brain Heart Infusion (BHI) broth/agar (Sigma-Aldrich); HiCrome E. coli agar (HiMedia); Agar (Sigma-Aldrich); Potassium permanganate ($KMnO_4$) agar. Solutions: Saline solution; Phosphate buffered solution (PBS); Electrolite saline solution w/ added lysozyme.

3. Isolation, cultivation and storage of the tested microorganisms

Isolation of pure cultures of oral lactic acid bacteria. Cultivation of the new isolates in MRS broth and agar at an optimal temperature. Storage of the new isolates in a frozen state at -80°C and under freeze dried state.

4. Physiological, genetic and bioinformatics methods

Gram staining; Peroxidase activity; Catalase activity.

Isolation of genomic DNA with Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research); Identification based on the 16S rDNA gene sequencing analysis with PuReTaq Ready-To-Go PCR Beads (Cytiva) and universal primers 27F and 1492R, sequencing of the PCR products by Macrogen Europe B. V., The Netherlands. The generated sequences were analysed by the BLASTN algorythm in the NCBI database; Whole genome sequencing (WGS) by Novogene Europe, United Kingdom. The raw data was processed with the Galaxy Europe bioinformatics platform and analysed with the MiGa Online platform for comparison with the NCBI Type Culture and Prokaryotic Genome Databases; Acquiring the protein profile with MALDI-TOF MS QuanTOF (IntelliBio) system.

In silico bioinformatics processing of the whole genome sequences for the presence of adhesins and lectins with the Galaxy Europe bioinformatics platform and BLASTx algorithm in the NCBI database; screening for acquired antibiotic resistance genes in the NCBI database; screening for peptidse activity genes in the NCBI database; screening for bacteriocin production genes in the NCBI database.

Enzymatic activity of the tested strains with API® ZYM strips (BioMérieux) test kit.

5. Survival Ability of the tested LAB strains in Simulated Oral Conditions

Investigating the influence of a model saliva solution on the tested strains and determining the CFU/mL from the viable cell counting.

6. *In vitro* survival ability and growth dynamics of the tested strains in the presence of GIT stress factors

Survival ability in the presence of pepsin and pH 2 by determining the CFU/mL from the viable cell counting. Growth dynamics in the presence of different gastrointestinal tract (GIT) stress factors, including lysozyme, pancreatic enzymes and pH 7, and varying concentrations of bile salts.

7. Adhesive properties of the tested strains

Autoaggregation capability by determining the optical density (OD); *In vitro* mucin binding capability by determining the CFU/mL adhered cells; Self biofilm forming capability by the crystal violet method.

8. Antibiotic resistance of the tested strains

Phenotypic determination of the antibiotic sensitivity by the Kirby-Bauer disc-diffusion method. Interpretation of the results is according to CLSI 2020.

9. Antioxidant capacity of the tested strains

Total antioxidant capacity by the potassium permanganate ($KMnO_4$) agar method.

10. Antimicrobial interactions of the tested strains

Antibacterial activity against eight test-pathogens *B. cereus*, *B. subtilis*, *E. coli*, *P. acnes*, *P. aeruginosa*, *S. aureus*, *S. epidermidis* and *S. mutans*; Antimicrobial activity against yeast test-pathogen *C. albicans*; Antagonistic activity against two test-pathogens *S. mutans* and *C. albicans* associated with oral diseases by the agar spot assay for qualitative inhibitory potential and direct antagonism cultivation for quantitative inhibitory potential; Coaggregation with *S. mutans* and *C. albicans* by determining the optical density (OD); *In vitro* antibiofilm activity against *S. mutans* and *C. albicans* by the crystal violet method.

11. Technological characteristics of the tested strains

Evaluation of the stability during freeze-drying process with two lyoprotective media and determining the CFU/mL from the viable cell counting before and after freeze-drying, and during 4 and 8 months of storage at 4°C; Evaluation of the stability during freeze-drying process with constructed lyoprotective medium for inclusion in the composition of a model product and determining the CFU/mL from the viable cell counting before and after freeze-drying.

12. Formulation of model products with selected strains for oral administration

Evaluating the probiotic potential and selection of strains to be included in the composition of model products; Formulation of cocoa and agar based model products with selected strains in fresh and freeze-dried state. Samples were stored at 4°C and analysed at 0, 10, 20, 30, 60 and 90 days by determining the CFU/mL from the viable cell counting; Presence of side microflora in the prepared model products by determining the total number of saprophytic microorganisms and the presence of *E. coli*.

13. Data analysis

Experiments were performed in triplicate and results were expressed as mean ± SD. Statistical analyses: Two-tailed Student's t-test; Pearson correlation; One-way ANOVA plus post-hoc Tukey test.

IV. Results

1. Isolation and characterization of LAB strains from the human oral cavity

1.1. Evaluation of morphological and physiological characteristics

The probiotic properties of LAB are widely studied, so they can be used as probiotic supplements for human healthcare. Despite the established characteristics of many established LAB strains, many of the current research focuses on new isolates regarding their different range of use (Un-Nisa et al., 2023).

In total, 76 samples were taken from the oral cavity of 16 volunteers without any serious oral diseases. 64 isolates were isolated from the samples and assessed with a classic sample processing approach. All isolates were evaluated for peroxidase and catalase enzymatic activities, cell morphology, and Gram staining. According to these criteria, twelve strains were preselected, showing negative peroxidase and catalase activities and were observed as Gram-positive rod-shaped cells (Table 1; Figure 1). From these results, all of the selected strains were defined as belonging to the group of LAB.

Table 1 – Morphological and physiological characteristics for preliminary selection of new isolates from the human oral cavity

Isolate	Cell morphology	Gram staining	Peroxidase activity	Catalase activity
N 2	rod-shaped	Gram+	-	-
N 4-5	rod-shaped	Gram+	-	-
AG 2-6	rod-shaped	Gram+	-	-
KG 12-1	rod-shaped	Gram+	-	-
TC 3-11	rod-shaped	Gram+	-	-
VG 1	rod-shaped	Gram+	-	-
VG 2	rod-shaped	Gram+	-	-
MK 13-1	rod-shaped	Gram+	-	-
NN 1	rod-shaped	Gram+	-	-
NA 1-8	rod-shaped	Gram+	-	-
NA 2-2	rod-shaped	Gram+	-	-
AV 2-1	rod-shaped	Gram+	-	-

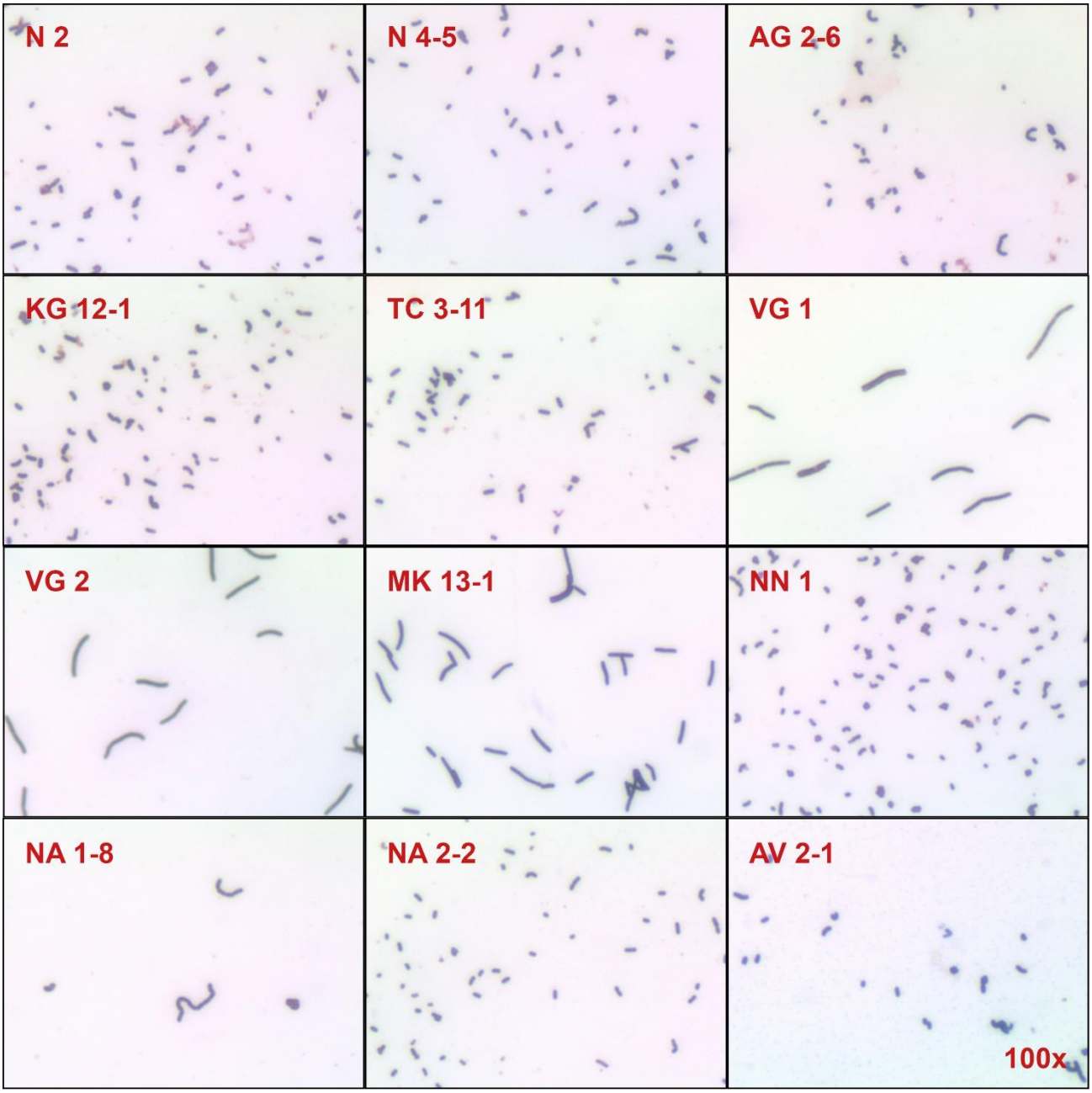

Figure 1 – Cell morphology of the new oral isolates

1.2. Screening for enzymatic activities of the newly isolated LAB strains

It is important for potential probiotics to be evaluated for the production of certain enzymes to confirm that they do not produce toxic substances. The production of useful enzymes and the inhibition of harmful enzymes exhibit different characteristics, which is dependent on the species of the tested microorganism. Therefore, species that produce potentially toxic substances need to be excluded. The API® ZYM system was used to obtain the enzymatic profiles of the studied strains (Table 2).

Table 2 – Enzymatic activity of the tested LAB strains.

Ензим	N 2	N 4-5	AG 2-6	KG 12-1	TC 3-11	VG 1	VG 2	MK 13-1	NN 1	NA 1-8	NA 2-2	AV 2-1
Alkaline phosphatase	1	1	2	2	1	0	0	0	0	1	1	3
Esterase (C4)	2	3	1	3	2	1	2	2	3	3	3	2
Esterase lipase (C8)	2	3	1	2	2	1	1	2	2	2	2	2
Lipase (C14)	1	2	1	1	1	1	1	1	1	2	2	2
Leucine arylamidase	5	5	5	5	5	5	5	5	5	5	5	5
Valine arylamidase	3	3	2	5	3	3	2	2	3	5	3	5
Cystine arylamidase	3	3	2	2	3	2	2	2	2	3	2	3
Trypsin	0	0	0	0	0	1	0	0	0	0	0	0
α-chymotrypsin	0	0	3	3	0	4	4	4	4	4	4	3
Acid phosphatase	5	4	5	5	4	3	3	3	4	3	3	4
Naphtol-AS-Biphosphohydrolase	1	1	4	5	1	4	2	3	5	4	4	2
α-galactosidase	4	5	2	1	5	4	3	3	5	3	5	0
β-galactosidase	5	5	1	5	5	2	2	4	5	3	5	5
β-glucuronidase	0	0	0	0	0	0	0	0	0	0	0	0
α-glucosidase	0	0	4	5	5	5	5	5	4	5	3	5
β-glucosidase	0	0	1	3	0	3	3	3	4	3	1	3
N-acetyl-β-glucosaminidase	0	0	0	1	0	0	0	0	0	0	0	1
α-mannosidase	0	0	0	0	0	0	0	0	0	0	0	0
α-fucosidase	0	0	0	1	0	0	0	0	0	1	0	0

All tested LAB strains exhibited high enzymatic activity for leucine arylamidase (5 according to API® ZYM scale) and acid phosphatase (3–5). Valine arylamidase, α-chymotrypsin, α- and β-galactosidase, and α-glucosidase activity (3–5) was reported for most of the strains. Low lipase (C14) activity (1–2) was exhibited by all of the strains and no enzymatic activity (0–1) was observed for the production of trypsin, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase.

In a study by Kim et al., the *L. paracasei* and *L. rhamnosus* strains included in their work showed high leucine arylamidase activity and no β-glucuronidase or α-mannosidase activity (Kim et al., 2021). The expression of arylamidase activities determines the proteolytic activity of the bacterial strains (Mudryk and Podgórska, 2006). The high leucine arylamidase activity of the tested LAB strains and the well-expressed strain-specific valine and cystine arylamidase activities qualifies their proteolytic activity.

It must be noted that β-glucuronidase is a bacterial enzyme associated with the production of certain harmful carcinogens and negative effects in the colon and liver (Song et al., 2020). According to the results, all of the tested strains did not exhibit β-glucuronidase enzymatic activity. Primary screening of basic enzymatic activities also contributes to the preliminary selection of potential probiotic strains, as these strains should not exhibit undesirable activities.

2. Species identification of the newly isolated LAB strains

New bacterial isolates with the potential to be used as probiotics need to be fully characterized (Sanders, 2008b). To be considered as probiotic candidates, the genera and species of the microorganisms must be identified as per the internationally accepted methods and nomenclature (Parker et al., 2019).

A polyphasic taxonomic approach for species identification of the newly isolated LAB strains was performed, including 16S rDNA gene sequencing analysis, *in silico* bioinformatics whole genome sequencing and processing, and acquiring the protein profile by the MALDI-TOF MS system.

Comparing the 16S rDNA gene sequences in the NCBI database showed a high percent of identity, above 98.7 %, for all twelve strains. The subsequent identification of the whole genome sequences of the tested strains also showed high percent of identification, above 97.9 %. Regardless of the percentage differences in the two approaches for each of the strains, the length of the nucleotide sequences must be taken into account both for the 16S rDNA gene and the whole genome sequence, which places the results of both approaches with high confidence of identification. This identification was verified by the PMF matching of the MALDI-TOF MS, measured by the score number of matching protein profiles. According to the QuanID Microbial Test application database, an interpretation of the identification score resulting in a score above >1000 is highly confident (Table 3). In addition, after the polyphasic taxonomic identification, the processed data of the whole genomic sequences of the tested LAB strains was deposited in the NCBI genetic database for whole genomes (Table 4; https://www.ncbi.nlm.nih.gov/bioproject/PRJNA991968).

Table 3 – Polyphasic taxonomic identification of the newly isolated LAB strains

Strain	16S rDNA sequencing	%	Whole genome sequencing	%	MALDI-TOF MS	Score
N 2	*Limosilactobacillus fermentum*	99.7	*Limosilactobacillus fermentum*	99.1	*Limosilactobacillus fermentum*	1586
N 4-5	*Limosilactobacillus fermentum*	99.6	*Limosilactobacillus fermentum*	99.1	*Limosilactobacillus fermentum*	1822
AG 2-6	*Weissella confusa*	99.4	*Weissella confusa*	97.9	*Weissella confusa*	1837
KG 12-1	*Latilactobacillus curvatus*	99.6	*Latilactobacillus curvatus*	98.8	*Latilactobacillus curvatus*	1189
TC 3-11	*Limosilactobacillus fermentum*	99.6	*Limosilactobacillus fermentum*	99.3	*Limosilactobacillus fermentum*	1234
VG 1	*Lactobacillus delbrueckii* subsp. *sunkii*	98.9	*Lactobacillus delbrueckii*	98.3	*Lactobacillus delbrueckii*	1324
VG 2	*Lactobacillus delbrueckii* subsp. *lactis*	98.7	*Lactobacillus delbrueckii* subsp. *lactis*	98.3	*Lactobacillus delbrueckii*	1676
MK 13-1	*Lactobacillus delbrueckii* subsp. *lactis*	99.1	*Lactobacillus delbrueckii* subsp. *lactis*	98.8	*Lactobacillus delbrueckii*	1193
NN 1	*Weissella confusa*	99.5	*Weissella confusa*	98.0	*Weissella confusa*	1490
NA 1-8	*Lacticaseibacillus rhamnosus*	99.9	*Lacticaseibacillus rhamnosus*	99.2	*Lacticaseibacillus rhamnosus*	1324
NA 2-2	*Limosilactobacillus fermentum*	99.1	*Limosilactobacillus fermentum*	99.5	*Limosilactobacillus fermentum*	2774
AV 2-1	*Lacticaseibacillus paracasei*	99.8	*Lacticaseibacillus paracasei*	98.6	*Lacticaseibacillus paracasei*	5533

Table 4 – Deposited whole genome sequences of the tested LAB strains

Strain	BioSample accession	Genome accession
Limosilactobacillus fermentum N 2	SAMN36329510	JAUJFN000000000
Limosilactobacillus fermentum N 4-5	SAMN36329511	JAUJFO000000000
Weissella confusa AG 2-6	SAMN36329512	JAUJFP000000000
Latilactobacillus curvatus KG 12-1	SAMN36329513	JAUJFQ000000000
Limosilactobacillus fermentum TC 3-11	SAMN36329514	JAUJFR000000000
Lactobacillus delbrueckii subsp. *sunkii* VG 1	SAMN36329515	JAUJFS000000000
Lactobacillus delbrueckii subsp. *lactis* VG 2	SAMN36329516	JAUJFT000000000
Lactobacillus delbrueckii subsp. *lactis* MK 13-1	SAMN36329517	JAUJFU000000000
Weissella confusa NN 1	SAMN36329518	JAUJFV000000000
Lacticaseibacillus rhamnosus NA 1-8	SAMN36329519	JAUJFW000000000
Limosilactobacillus fermentum NA 2-2	SAMN36329520	JAUJFX000000000
Lacticaseibacillus paracasei AV 2-1	SAMN36329521	JAUJFY000000000

The twelve strains were identified as representatives from different LAB species from the *Lactobacillaceae* family. According to the newly proposed nomenclature for the *Lactobacillus* genera (Zheng et al., 2020), the strains were identified as *Limosilactobacillus fermentum*, *Latilactobacillus curvatus*, *Lactobacillus delbrueckii* subsp. *sunkii*, *Lactobacillus delbrueckii* subsp. *lactis*, *Lacticaseibacillus rhamnosus*, *Lacticaseibacillus paracasei*, and *Weissella confusa* (Table 3).

Determining the nucleotide sequence of the 16S rDNA gene and the whole genome sequences allows the phylogenetic analysis and identification of significant clinical and industrial strains (Salem-Bango et al., 2023). Although identifying microorganisms using the 16S rDNA is the most used method, in recent years the MALDI-TOF MS has proved to be a very promising tool for identification, classification, and diagnosis (Singhal et al., 2015) and is being widely used (Jussiaux et al., 2021). The obtained results from the MALDI-TOF MS analyses provided a highly accurate verification of the genetic identifications of the twelve newly isolated LAB strains (Table 3).

In addition to the results from the enzymatic activities, different genes for peptidase activity were determined from the *in silico* analyses of the tested strains. The detected genes are from the Pep family of hydrolase enzymes which hydrolyse different proteins, including dipeptides, tripeptides and oligopeptides, to aminoacids (Table 5). LAB provide their cells with essential aminoacids for their growth process by exhibiting hydrolase activity and can produce different substances that are beneficial to human health (Wang et al., 2021).

Table 5 – Determined peptidase activity genes for the tested LAB srains

Strain	pepA	pepC	pepF	pepQ	pepT	pepV
L. fermentum N 2			+		+	+
L. fermentum N 4-5			+		+	+
W. confusa AG 2-6	+		+		+	+
L. curvatus KG 12-1			+		+	+
L. fermentum TC 3-11					+	+
L. delbrueckii subsp. sunkii VG 1		+	+	+	+	+
L. delbrueckii subsp. *lactis* VG 2			+		+	
L. delbrueckii subsp. *lactis* MK 13-1		+	+	+	+	+
W. confusa NN 1	+		+		+	+
L. rhamnosus NA 1-8						+
L. fermentum NA 2-2			+		+	+
L. paracasei AV 2-1			+		+	+

The endopeptidase system of LAB consists of aminopeptidases (PepN, PepC, PepM, and PepA), endopeptidases (PepE, PepF, and PepO), dipeptidases (PepV and PepD), the tripeptidase PepT, proline peptidases (PepX, PepP, PepQ, PepR, and PepI). Many peptides with essential physiological functions were found in casein hydrolyzates, such as opioid peptide, blood pressure lowering peptide, antithrombotic peptide, immunostimulant peptide, mineral ions absorbtion promoting peptide, etc. These peptides are around 1000 Da and can be easily absorbed in the GIT (Dimitrov et al., 2015; Zhang et al., 2022). The presence of

peptidase activities is essential characteristic which has potential for the production of biologically active peptides and complements the probiotic properties of the different LAB strains.

3. Evaluation of survival ability and growth dynamics of the tested LAB strains in GIT

3.1. Survival ability in simulated oral conditions

The oral cavity is the entry point of the GIT and the received probiotic microorganisms are initially exposed to the saliva. During this first contact with the host, the bacterial survival ability of the oral environment is of utmost importance. The protein components of the saliva, including lysozyme, salivary peroxidase, lactoferin, histatin, cystatines, and secretory IgA can affect the viability or activity of the probiotic strains, such as the adhesion and metabolic activity. The influence of saliva on the microbial establishment can, on the one hand, inhibit microbial colonization, but on the other, it can promote it (Bosch et al., 2003). Preliminary treatment of LAB with lysozyme can slightly enhance adhesion but can also slightly lower their survival ability (Stamatova et al., 2009).

To evaluate the survivability of LAB in the human body, in vitro analyses for their resistance to the conditions of the GIT were made. The purpose of the survival assessment in simulated oral conditions was to determine the effect of the saliva-resembling electrolyte saline solution with added lysozyme on the survival of the studied LAB strains when they enter the oral cavity. It can be observed that after treatment with the electrolyte saline solution with added lysozyme, all tested LAB strains maintain the same log CFU/mL as their untreated samples (Figure 2).

A study by Haukioja et al. evaluated the survivability in saliva and an adhesion to the oral surfaces of *Lactobacillus* and *Bifidobacterium* probiotics. All tested strains showed a high survivability, but their adhesion varied, as the Lactobacillus species expressed high adhesion properties (Haukioja et al., 2006). *In vitro* studies report that *Lactobacillus* and *Bifidobacterium* cannot grow in saliva but they can stay viable for 24 hours after incubation. The preliminary treatment with lysozyme significantly decreased the adhesion of *L. rhamnosus* GG, *L. rhamnosus* Lc705, and *L. casei* Shirota, but the adhesion properties of *Lactobacillus johnsonii* La1 and *Bifidobacterium lactis* Bb12 remained unchanged (Haukioja et al., 2008).

Since all tested LAB strains are oral isolates and their natural environment is the oral cavity, it is expected that they can sustain the effect of the saliva. The recorded data show that when treated with the saliva-resembling solution, all of the tested strains show no significant change in CFU/mL compared with the control samples. This points a high survivability potential in a saliva environment, which is a prerequisite for their ability to compete with pathogens and to be a part of the dental biofilm and exhibit their probiotic properties under the conditions of the oral cavity.

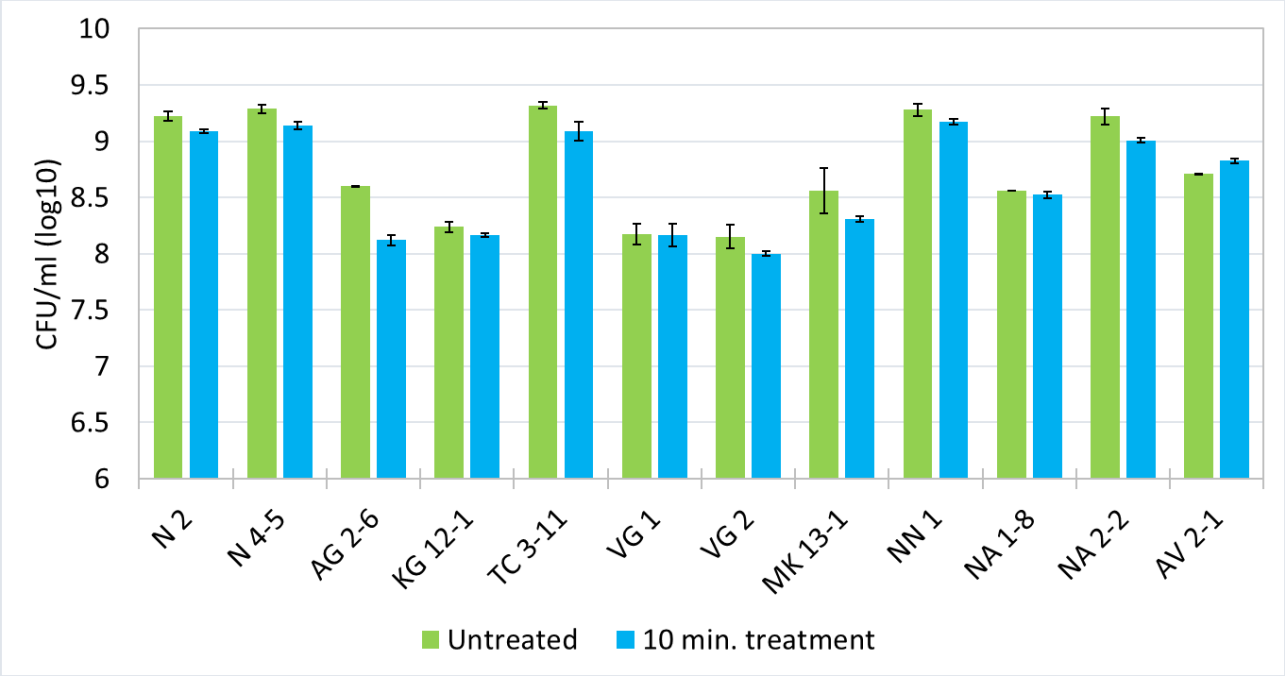

Figure 2 – Survival ability of the newly isolated lactic acid bacterial strains under simulated oral stress conditions. Values are expressed as mean ± standard deviation (SD). Two-tailed Student's t-test: a – $p > 0.05$; b and c – $p < 0.05$ and $p < 0.01$, respectively. Strains: *L. fermentum* N 2, *L. fermentum* N 4-5, *W. confusa* AG 2-6, *L. curvatus* KG 12-1, *L. fermentum* TC 3-11, *L. delbrueckii* subsp. *sunkii* VG 1, *L. delbrueckii* subsp. *lactis* VG 2, *L. delbrueckii* subsp. *lactis* MK 13-1, *W. confusa* NN 1, *L. rhamnosus* NA 1-8, *L. fermentum* NA 2-2, and *L. paracasei* AV 2-1.

3.2. Survival ability under direct exposure to stress factors in GIT

The transfer of a probiotic from the oral cavity to the next parts of the GIT is very likely, so it is important that their ability to survive in this transition is studied as well. Microorganisms that enter the upper and lower parts of the GIT are influenced by the presence of different enzymes. For potential probiotic LAB, it is required that they can resist the stressful factors of the GIT to exhibit their probiotic properties.

The purpose of the analysis was to evaluate the stability of the tested LAB strains upon exposure to the conditions of the gastric environment. The resistance to pepsin in pH 2 was held for 3 h at 37 °C and the log values of CFU/mL from the viable cell count was reported. The results show that most of the tested strains exhibit a decreased cell viability by 2–3 logs (Figure 3). For *L. fermentum* N 2, N 4-5, TC 3-11, NA 2-2 and *L. delbrueckii* subsp. *lactis* MK 13-1, and *L. rhamnosus* NA 1-8, a decrease of about 2 logs is observed. For *W. confusa* AG 2-6 and NN 1, and *L. delbrueckii* subsp. *lactis* VG 2, a decrease of up to 3 logs is observed for the duration of the experiment. The strains *L. curvatus* KG 12-1, *L. delbrueckii* subsp. *sunkii* VG 1, and *L. paracasei* AV 2-1 show no survivability in a simulated harsh environment of the stomach in the presence of pepsin in pH 2.

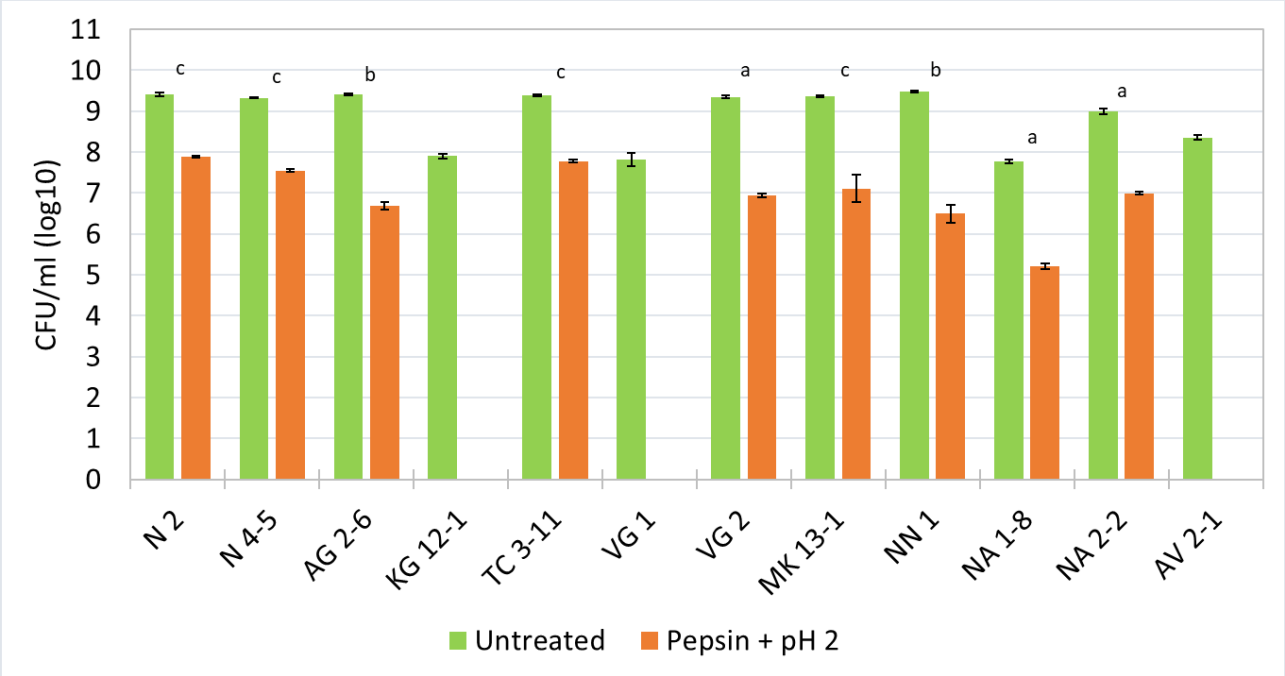

Figure 3 – Survival ability of the newly isolated lactic acid bacterial strains in pepsin in pH 2. Values are expressed as mean ± SD. Two-tailed Student's t-test: a – $p > 0.05$; b and c – $p < 0.05$ and $p < 0.01$, respectively. Strains: *L. fermentum* N 2, *L. fermentum* N 4-5, *W. confusa* AG 2-6, *L. curvatus* KG 12-1, *L. fermentum* TC 3-11, *L. delbrueckii* subsp. *sunkii* VG 1, *L. delbrueckii* subsp. *lactis* VG 2, *L. delbrueckii* subsp. *lactis* MK 13-1, *W. confusa* NN 1, *L. rhamnosus* NA 1-8, *L. fermentum* NA 2-2, and *L. paracasei* AV 2-1.

The low pH values in the stomach and the antimicrobial effect of the digestive enzyme pepsin act as a barrier for LAB to survive in the GIT. Because their natural origin is the oral cavity, it is possible that some strains might not withstand very low pH values and the presence of pepsin. The results from this experiment indicate that nine of all the tested strains show a considerable survival ability, which highly favors their possibility to withstand the harsh environment of the stomach and transit to the next sections of the GIT.

Many authors report an expressed sensitivity of many LAB strains under the conditions of the enzymatic activity and high acidity of the stomach environment. Mantzourani et al. reported a decreased viability of 2–5 logs observed within the range of the tested LAB isolates (Mantzourani et al., 2019). A study by Tokatlı et al. evaluated the survivability of several LAB strains and reported a decrease in the cell viability of up to 4 logs (Tokatlı et al., 2015).

3.3. Growth dynamics in the presence of GIT stress factors

In order to survive and maintain their activity in the oral cavity, as well as in GIT, potential probiotc LAB must exhibit resistance to the stressful environment in GIT. Factors like lysozyme, pancreatin, bile salts, and ranging pH levels can influence the survival ability and growth of LAB. Series of experiments were carried out in order to evaluate the growth ability of the tested strains in the presence of different stress factors.

The growth dynamics in the presence of lysozyme were assessed for 24 h at 37 °C and the OD was measured every 2 hours at 600 nm. The results are shown in Figures 4.1 and 4.2 as growth curves. Eight of all the tested strains exhibit similar growth rates as their control samples, except the strains *L. curvatus* KG 12-1, *L. delbrueckii* subsp. *sunkii* VG 1, and *L. delbrueckii* subsp. *lactis* VG 2 and MK 13-1. Although the media with added lysozyme influences their growth, they show a high survival ability in these conditions (Figure 2).

Being of an oral origin, the newly isolated LAB strains show high adaptive properties to the presence of this enzyme and have the potential to persist in the environment of the oral cavity and their beneficial properties to be applied. Studies by Fang et al. and Jia et al. reported high tolerance levels when cultivating *Lactobacillus* strains in the presence of different concentrations of added lysozyme (Fang et al., 2018; Jia et al., 2019). Bosch et al. evaluated the effect of different concentrations of lysozyme on the growth of LAB strains and reported growth ranges of 95.86–49.12% (Bosch et al., 2012).

The growth dynamic of the studied strains in the presence of pancreatin in pH 7 were assessed (Figures 4.1; 4.2). The results show that the pancreatic enzymes in neutral pH do not decrease the growth of the tested strains. The observed growth properties in the presence of pancreatin makes the adaptivity of the tested strains highly possible in these conditions. Khagwal et al. reported a resistance to the presence of pancreatin in a concentration of 0.5% for all tested LAB strains in their study (Khagwal et al., 2014).

The growth dynamic in the presence of bile salts were assessed to evaluate the effect of different concentrations of bile salts on the studied strains (Figures 4.1; 4.2). The presence of 0.3% bile salts decreases the cell growth for the strains *L. fermentum* N 2, N 4-5, TC 3-11, and NA 2-2, *W. confusa* AG 2-6 and NN 1, and *L. paracasei* AV 2-1 and a lower cell density is reported at the end of the experiment in comparison with the control variants. While for the strains *L. curvatus* KG 12-1, *L. delbrueckii* subsp. *sunkii* VG 1, *L. delbrueckii* subsp. *lactis* VG 2 and MK 13-1, and *L. rhamnosus* NA 1-8, growth inhibition is observed. Bile salts in higher concentrations of 0.5% and 1.0% significantly inhibit the growth of all LAB strains. It is necessary to specify that in physiological conditions, the bile salts concentration of a healthy person is no more than 0.3% (Jose et al., 2015), so these results indicate that seven of the twelve tested strains can overcome the effect of bile salts in a concentration of 0.3% and exhibit growth in this environment, which makes these strains able to withstand the normal conditions of the colon. The effect of the bile concentration is reported to be species- and strain-dependent (Montville and Matthews, 2012). A study by Alameri et al. reported a 50–60% growth rate in ox-bile medium after 6 hours, compared with the control samples, for most of the LAB strains used in their work (Alameri et al., 2022). A Lactobacillus strain evaluated by Aarti et al. showed a high tolerance for 36 hours of cultivating in a bile salts medium (Aarti et al., 2018).

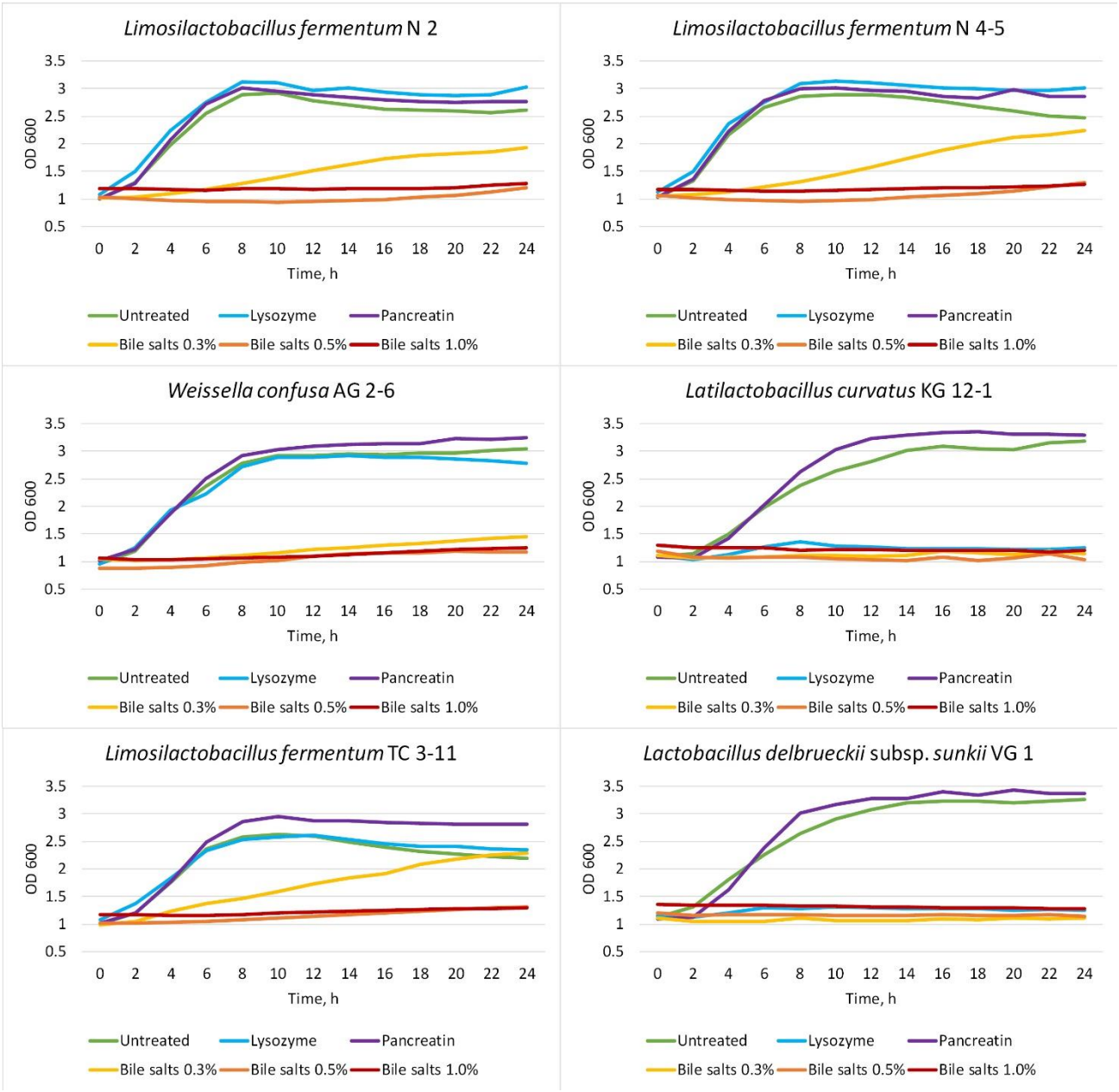

Figure 4.1 – Growth dynamics of the newly isolated LAB strains in the presence of gastrointestinal stress factors.

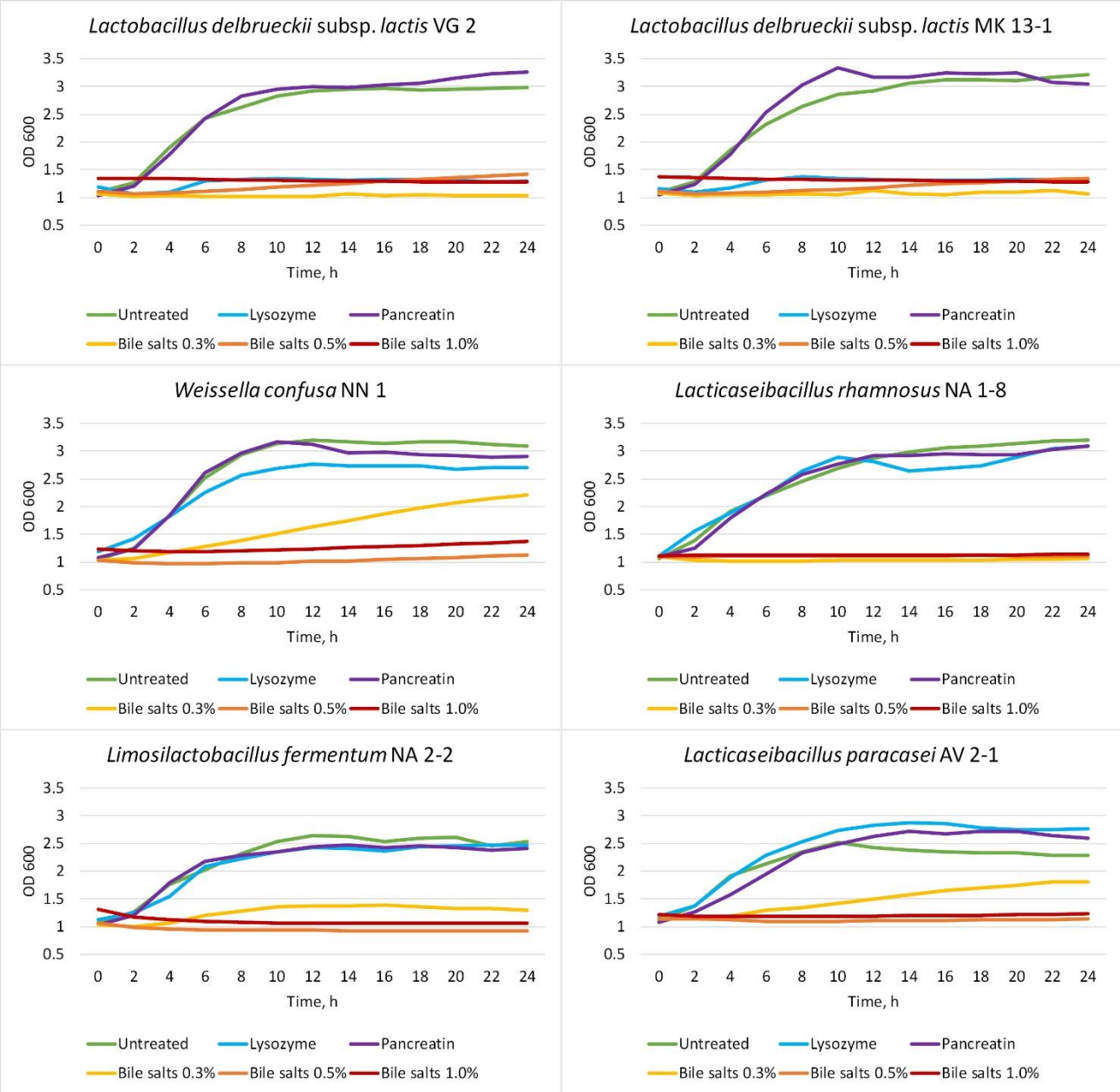

Figure 4.2 – Growth dynamics of the newly isolated LAB strains in the presence of gastrointestinal stress factors.

To summarise, the results from the evaluated survival ability and growth dynamics experiments in the presence of different GIT stress factors point that these characteristics are not species determined, but are strain dependant.

4. Evaluation of the adhesive properties of the tested LAB strains

4.1. Autoaggregation capability

The ability to adhere to epithelial cells in the GIT is one of the main characteristics of LAB, by which they exert their beneficial effects on the host's health state. Observed adhesion properties can promote for increased residence time of LAB in the GIT, preventing potential pathogens from surface space and their elimination from the dental biofilm, and providing protective functions for the epithelial cells (Guan et al., 2020).

The autoaggregative potential of the tested LAB strains was assessed and presented in Figure 5. The results show that all the tested strains can autoaggregate and exhibited these properties in the range of 10.3–26.6% for 4 hours of incubation. The highest autoaggregation was reported for the trains *L. delbrueckii* subsp. *sunkii* VG 1, *L. rhamnosus* NA 1-8 and *L. paracasei* AV 2-1. All other LAB strains exhibited below 20% autoaggregative properties. It is important to note that the conditions of the experiment, such as the culture age, time, and temperature, have a significance for the determination of the autoaggregation ability.

The ranging autoggregation levels that are observed amongst the LAB strains result from a complex interaction between different bacterial cell surface molecules, such as proteins and polysaccharides (Li et al., 2020), and this property is considered to be strain-specific (Trunk et al., 2018). In other studies, 22 *Lactobacillus* strains were tested that showed autoaggregative properties in the range of 24 to 41% (Tuo et al., 2013) and several Lactobacillus strains that exhibited autoaggregation in the range of 15 to 21% (Campana et al., 2017).

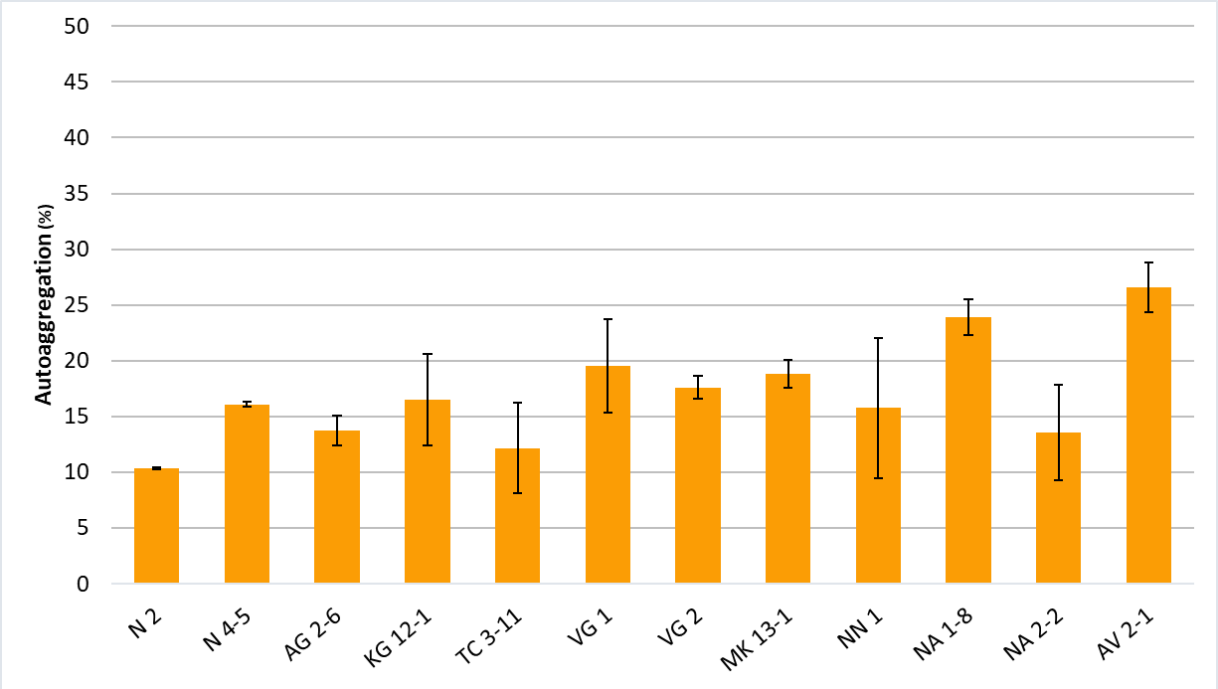

Figure 5 – Autoaggregation of the newly isolated LAB strains. Values are expressed as mean ± SD. Strains: *L. fermentum* N 2, *L. fermentum* N 4-5, *W. confusa* AG 2-6, *L. curvatus* KG 12-1, *L. fermentum* TC 3-11, *L.*

delbrueckii subsp. *sunkii* VG 1, *L. delbrueckii* subsp. *lactis* VG 2, *L. delbrueckii* subsp. *lactis* MK 13-1, *W. confusa* NN 1, *L. rhamnosus* NA 1-8, *L. fermentum* NA 2-2, and *L. paracasei* AV 2-1.

4.2. Mucin binding capability

For evaluation of the adhesion properties of the tested LAB strains to the mucosal tissue, a mucin binding *in vitro* analysis was assessed. The results show that all of the tested LAB strains exhibit an expressed ability to bind to mucin, measured at 5 logs CFU/mL (Figure 6).

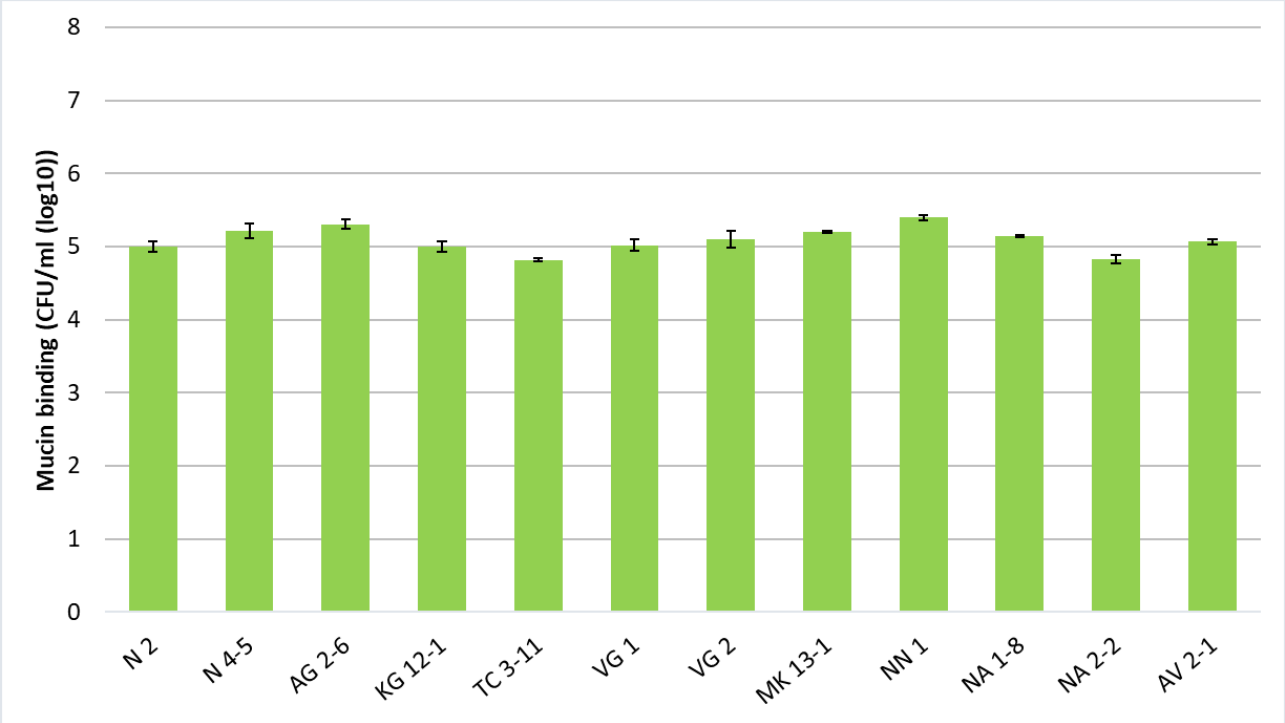

Figure 6 – Mucin binding capability of the newly isolated LAB strains. Values are expressed as mean ± SD. Strains: *L. fermentum* N 2, *L. fermentum* N 4-5, *W. confusa* AG 2-6, *L. curvatus* KG 12-1, *L. fermentum* TC 3-11, *L. delbrueckii* subsp. *sunkii* VG 1, *L. delbrueckii* subsp. *lactis* VG 2, *L. delbrueckii* subsp. *lactis* MK 13-1, *W. confusa* NN 1, *L. rhamnosus* NA 1-8, *L. fermentum* NA 2-2, and *L. paracasei* AV 2-1.

Exhibiting an adhesion to mucin makes probiotic LAB able to adapt to the environments of GIT, colonize inside the host, and exert their beneficial properties. The assessed adhesion of the tested LAB strains suggests that they possess a capacity for an *in vivo* colonization and the obtained results have an important relevance for the probiotic use of these strains.

A study by Monteiro et al. reported similar mucin binding properties for *L. plantarum* and *L. fermentum* strains (Monteiro et al., 2019).

The ability of the tested strains to adhere to mucin proteins predetermines their potential to successfully adapt and colonize in the different compartments of the GIT and exhibit their probiotic properties. The evaluated adhesion ability of the tested strains determines their potential for *in vivo* colonization and the obtained results are important to define their probiotic potential, and their future application in probiotic products. Being of oral origin, all tested strains possess the potential ability to adhere to oral mucosa, be included in the oral microbiome, and exhibit their probiotic properties.

4.3. Biofilm formation ability

The formation of a biofilm is an important mechanism by which LAB adhere and accumulate on the oral tissues and can successfully exhibit their probiotic properties. This characteristic also enhances their antagonistic effect against pathogenic microorganisms in GIT (Mgomi et al., 2023).

The crystal violet staining assay can be used as an indirect method for determining the amount of accumulated bacterial biofilm (Djordjevic et al., 2002). All tested LAB strains showed the ability to form biofilms with differentiating percent (Figure 7). *L. delbrueckii* subsp. *lactis* VG 2 expressed a percentage of biofilm formation above 96%, the highest among the tested LAB strains in this *in vitro* analysis. For *L. fermentum* TC 3-11 and N 4-5 strains, well-expressed formation of biofilm was also reported, measured above 74%. For *L. fermentum* NA 2-2, *L. delbrueckii* subsp. *sunkii* VG 1 and *L. delbrueckii* subsp. *lactis* MK 13-1 self biofilm formation ability was exhibited above 50%.

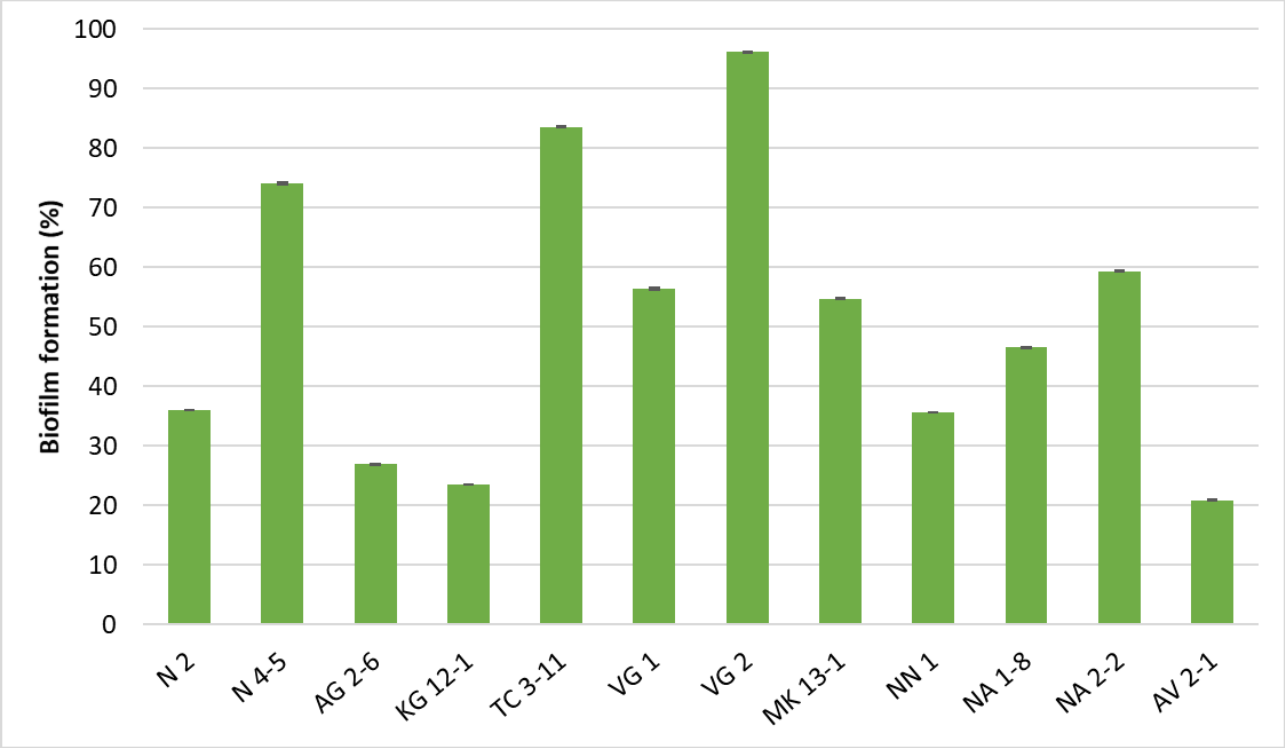

Figure 7 – Percent of biofilm formation by the tested LAB strains. Values are expressed as mean ± SD. Statistical analysis was performed by One-way ANOVA ($p < 0.01$). Strains: *L. fermentum* N 2, *L. fermentum* N 4-5, *W. confusa* AG 2-6, *L. curvatus* KG 12-1, *L. fermentum* TC 3-11, *L. delbrueckii* subsp. *sunkii* VG 1, *L. delbrueckii* subsp. *lactis* VG 2, *L. delbrueckii* subsp. *lactis* MK 13-1, *W. confusa* NN 1, *L. rhamnosus* NA 1-8, *L. fermentum* NA 2-2, and *L. paracasei* AV 2-1.

The results from the biofilm formation assay show significant differences among strains of the same species. Biofilm formation among the four *L. fermentum* strains ranges between 36% and 83%. From the tested *L. delbrueckii* strains, while VG 1 and MK 13-1 exhibited

similar biofilm formation, for VG 2, the formed biofilm was noticeably higher. The two *W. confusa* strains AG 2-6 and NN 1 also showed different percentages of biofilm formation. This data suggests biofilm formation could be a strain-specific property among LAB representatives. This could also be influenced by environmental factors and microbial composition in the oral cavity.

In a study by Jha et al., the biofilm formation among the six tested LAB strains varied between 19.32 and 85.84% (Jha et al., 2022). Gómez et al. described similar strain dependency between the studied *L. lactis* and *L. curvatus* strains (Gómez et al., 2016). Kubota et al. evaluated 46 LAB strains, including eight *L. plantarum* and four *L. brevis* strains, showing differences in biofilm formation capability among the same species (Kubota et al., 2008).

4.4. *In silico* bioinformatics analysis for presence of adhesion proteins

Many bioinformatics tool for genetic processing are present, as well as analytical techniques for determination of molecular mechanisms responsible for recognition and adhesion of bacterial cells on mucosal surfaces. Such mechanisms also include carbohydrate-protein interactions through adhesins expressed on the surface of bacterial cells (Van Tassell and Miller, 2011; Juge, 2012; Etzold and Juge, 2014).

In silico bioinformatics analyses of the whole genome sequences were carried through for the presence of genetic determinants for adhesins responsible for the determined adhesion characteristics of the tested strains from the *in vitro* analyses. Two types of adhesion proteins were discovered in the bioinformatics processing: adhesins, which facilitate the adhesion of LAB to tissues and other cells, and lectins, which facilitate the binding of LAB to carbohydrates and other proteins (Table 6).

It was established that genetic determinants for adhesion expression are present in the tested LAB strains. They contain LPxTG, YSIRK, KxYKxGKxW, and SEC 10/PgrA domains, as well as MucBP domains that are a part of the mucin-binding protein family. On the other hand, lectins were found for *L. curvatus* KG 12-1, *L. rhamnosus* NA 1-8, *L. paracasei* AV 2-1, *W. confusa* AG 2-6 and NN 1 with the presence of WxL domain from the CscC protein family and L-type lectin domain.

Adhesins expressed by LAB are classified according to the target place for adhesion on the mucous tissue, as well as their localization and/or their binding to the cell surface (Vélez et al., 2007). The cell surface contains protein molecules in its structure, which are connected to the cell wall by a binding domain at the C-terminus, coded by an LPxTG sequence. Different domains with YSIRK *и* KxYKxGKxW sequences are bound at the N-terminus of the protein molecule which determine the adhesive properties of LAB, as well as their competitiveness to various pathogenic representatives. The SEC 10/PgrA domain determines the the ability to perform adhesion and the MucBP domain is responsible mainly for the binding of bacterial cells to the expressed mucin proteins on the mucosal surface (Nishiyama et al., 2016; Patel et al., 2017; Albarracin et al., 2022).

Table 6 – Detection of adhesins and lectins from the processing of the whole genome sequences of the tested LAB strains.

Strain	Adhesins	Lectins
L. fermentum N 2	KxYKxGKxW signal peptide domain-containing protein LPxTG cell wall anchor domain-containing protein MucBP domain-containing protein SEC 10/PgrA surface exclusion domain-containing protein YSIRK-type signal peptide-containing protein	-
L. fermentum N 4-5	KxYKxGKxW signal peptide domain-containing protein LPxTG cell wall anchor domain-containing protein SEC 10/PgrA surface exclusion domain-containing protein YSIRK-type signal peptide-containing protein	-
W. confusa AG 2-6	KxYKxGKxW signal peptide domain-containing protein LPxTG cell wall anchor domain-containing protein MucBP domain-containing protein	WxL domain-containing protein L-type lectin-domain-containing protein
L. curvatus KG 12-1	LPxTG cell wall anchor domain-containing protein MucBP domain-containing protein	WxL domain-containing protein
L. fermentum TC 3-11	KxYKxGKxW signal peptide domain-containing protein LPxTG cell wall anchor domain-containing protein MucBP domain-containing protein SEC 10/PgrA surface exclusion domain-containing protein YSIRK-type signal peptide-containing protein	-
L. delbrueckii subsp. *sunkii* VG 1	LPxTG cell wall anchor domain-containing protein SEC 10/PgrA surface exclusion domain-containing protein YSIRK-type signal peptide-containing protein	-
L. delbrueckii subsp. *lactis* VG 2	LPxTG cell wall anchor domain-containing protein SEC 10/PgrA surface exclusion domain-containing protein YSIRK-type signal peptide-containing protein	-
L. delbrueckii subsp. *lactis* MK 13-1	LPxTG cell wall anchor domain-containing protein SEC 10/PgrA surface exclusion domain-containing protein YSIRK-type signal peptide-containing protein	-
W. confusa NN 1	KxYKxGKxW signal peptide domain-containing protein LPxTG cell wall anchor domain-containing protein MucBP domain-containing protein	WxL domain-containing protein L-type lectin-domain-containing protein
L. rhamnosus NA 1-8	KxYKxGKxW signal peptide domain-containing protein LPxTG cell wall anchor domain-containing protein MucBP domain-containing protein	WxL domain-containing protein
L. fermentum NA 2-2	KxYKxGKxW signal peptide domain-containing protein LPxTG cell wall anchor domain-containing protein MucBP domain-containing protein SEC 10/PgrA surface exclusion domain-containing protein YSIRK-type signal peptide-containing protein	-
L. paracasei AV 2-1	LPxTG cell wall anchor domain-containing protein MucBP domain-containing protein SEC 10/PgrA surface exclusion domain-containing protein	WxL domain-containing protein L-type lectin-domain-containing protein

For LAB expressing lectins, the WxL domain is responsible for localization of cell surfaces and the L-type lectin domain is responsible for the interaction with the mucosal epithelial cells (Brinster et al., 2007; Tsuchiya et al., 2023). The detection of genetic determinants for adhesion proteins supports the established results for aggregation, mucin

binding, and biofilm formation *in vitro* analyses. Also, these cell surface structures could also play a role in co-aggregation with pathogens and in antibiofilm activity, which results are described in secion 9.

5. Evaluation of antibiotic resistance and bioinformatics analysis for acquired genes of the tested LAB strains

Evaluation of the antibiotic resistance is one of the main important standards related to the technological application and probiotic potential of LAB. Evaluating the antibiotic resistanceof LAB by applying phenotypic and genotypic analyses is based on determining the possible horizontal transfer of antibiotic resistance genes to commensal and pathogenic microorganisms, the relationship of some species in some disease cases, and the possibility of a combined antibiotic treatment with probiotic in order to restore the normal microflora in the GIT (Gad et al., 2014)

Thirteen antibiotics were assessed in the antibiotic resistance analysis of the tested strains. The used antibiotics were selected depending on their mechanism of action: inhibition of the protein synthesis, inhibition of the cell wall synthesis, and inhibition of the DNA synthesis (Table 7).

Susceptibility of all LAB strains to five of the tested antibiotics (ampicillin, clindamycin, chloramphenicol, erythromycin, and tetracyclin) was reported. Resistance to rifampicin was reported for the two *W. confusa* AG 2-6 and NN 1 strains. The tested strains exhibit expressed resistance to three of the tested antibiotics – kanamycin, neomycin, and streptomycin, which are inhibitors of the protein synthesis. Only the *L. fermentum* TC 3-11 strain showed intermediate susceptibility to gentamicin while all other strains are resistant. Only the *L. rhamnosus* NA 1-8 strain showed intermediate susceptibility to ciprofloxacin, which inhibits the DNA synthesis, while all other strains are resistant. The three *L. delbrueckii* strains showed intermediate susceptibility to vancomycin, which inhibits the cell wall synthesis, while all other strains are resistant. Resistance to trimethoprim was reported for most of the strains, except the *L. fermentum* N2, TC 3-11 and NA 2-2 strains which are susceptible to this antibiotic.

In addition, the multiple antibiotic resistance index was calculated, showing values between 0.385 and 0.615 for the different strains.

Table 7 – Antibiotic resistance of the tested LAB strains. Strains: *L. fermentum* N 2, *L. fermentum* N 4-5, *W. confusa* AG 2-6, *L. curvatus* KG 12-1, *L. fermentum* TC 3-11, *L. delbrueckii* subsp. *sunkii* VG 1, *L. delbrueckii* subsp. *lactis* VG 2, *L. delbrueckii* subsp. *lactis* MK 13-1, *W. confusa* NN 1, *L. rhamnosus* NA 1-8, *L. fermentum* NA 2-2, and *L. paracasei* AV 2-1.

Strain	Antibiotics													MAR index
	AMP	CD	VA	CIP	TR	C	E	GEN	K	N	RD	S	TE	
N 2	S	S	R	R	S	S	S	R	R	R	S	R	S	0.462
N 4-5	S	S	R	R	R	S	S	R	R	R	S	R	I	0.538
AG 2-6	S	S	R	R	R	S	S	R	R	R	R	R	S	0.615
KG 12-1	S	S	R	R	R	S	S	R	R	R	S	R	S	0.538
TC 3-11	S	S	R	R	S	S	S	I	R	R	S	R	S	0.385
VG 1	S	S	I	R	R	S	S	R	R	R	S	R	S	0.462
VG 2	S	S	I	R	R	S	S	R	R	R	S	R	S	0.462
MK 13-1	S	S	I	R	R	S	S	R	R	R	S	R	S	0.462
NN 1	S	S	R	R	R	S	S	R	R	R	R	R	S	0.615
NA 1-8	S	S	R	I	R	S	S	R	R	R	S	R	S	0.462
NA 2-2	S	S	R	R	I	S	S	R	R	R	S	R	S	0.462
AV 2-1	S	S	R	R	R	S	S	R	R	R	S	R	S	0.538

R – resistant, I – intermediate, S – susceptible (CLSI, 2020); Антибиотици: Ampicillin (AMP) – 10 mcg/disk; Clindamycin (CD) – 2 mcg/disk; Vancomycin (VA) – 5 mcg/disk; Ciprofloxacin (CIP) – 5 mcg/disk; Trimethoprim (TR) – 5 mcg/disk; Cloramphenicol (C) – 30 mcg/disk; Erythriomycin (E) – 15 mcg/disk; Gentamicin (GEN) – 10 mcg/disk; Kanamycin (K) – 30 mcg/disk; Neomycin (N) – 30 mcg/disk; Rifampicin (RD) – 5 mcg/disk; Streptomycin (S) – 10 mcg/disk; Tetracycline (TE) – 30 mcg/disk.

In addition to the phenotypic antibiotic resistance analysis, an in silico bioinformatics analyses for detection of acquired antibiotic resistance genes were held. The whole genome sequences were processed and the results show that the tested LAB strains do not possess acquired cat, erm(B), and tet(M) antibiotic resistance genes, which are one of the mainly tracked. This result allows the strains to be evaluated as safe under the Qualified Presumption of Safety (QPS) requirements of EFSA (EFSA, 2018).

The determined antibiotic resistance of the twelve tested strains is defined by different intracellular mechanisms (Anisimova and Yarullina, 2019). Most of the LAB representatives possess high resistance to glycopeptide antibiotic substances, including vancomycin, as this characteristic is determined by differences in the chromosome coding for the peptidoglycan assembly pathway (Zhang et al., 2018b). Also, LAB are mostly resistant to aminoglycoside antibiotics, including kanamycin, neomycin, streptomycin, and gentamicin, as this phenotypic characteristic is considered to be due to two main factors: low bacterial cell wall permeability to aminoglycosides and absence of elements of the cytochrome mediated electron transfer (Dec et al., 2018; Duskova et al., 2020). It is also reported that LAB which possess intrinsicresistance to diaminopyrimidines, including trimethoprim, do not possess biosynthetic pathway for production of folic acid, as the antibiotic blocks the dihydropteroat synthetase activity in the cells (Ammor et al., 2007; Duche et al., 2023). The resistance to fluoroquinolone antibiotics, including ciprofloxacin, is probably due to cell wall

impermeability, as well as mutations in the quinolone resistance regions which determine the genetic basis for antibiotic resistance for LAB (Li et al., 2015; Zarzecka et al., 2022).

From numerous studies, it is considered that the resistance to specific antibiotic groups, as well as the susceptibility to other depends on the source of origin of the isolates. It is hypothesized that human-derived LAB isolates exhibit more widespread resistance than those from food sources due to the higher likelihood of exposure to antibiotic substances in their natural environment (Danielsen and Wind, 2003; Sharma et al., 2017; Duche et al., 2023). The established resistances for the studied strains are also confirmed by other authors, including Delgado et al., 2005; Zhou et al., 2005; Selvin et al., 2020, and Wang et al., 2022, as in other of the previously cited references.

6. Antioxidant capacity screening of the tested LAB strains

Important and not as widely studied LAB property is their antioxidant activity. The antioxidant activity of LAB is essential to be studied as their antioxidant enzyme production can protect the host from damage from free radicals that have a role in the development of many chronic diseases (Zehiroglu and Ozturk Sarikaya, 2019). The used method is recently adapted for LAB and estimates the total antioxidant capacity of their native CFS (Hanchi et al., 2022).

All tested LAB CFSs exhibited antioxidant capacity throughout the experiment (Figure 8). The organic antioxidant compounds react with the $KMnO_4$, and distinct transparent zones can be observed. At 10 min, small occurring halo zones can already be seen. At 30 min, 1 h and 4 h, the zones have clear boundaries and the CFSs from *L. fermentum* TC 3-11 (pH 3.72) and NA 2-2 (pH 3.92), *L. delbrueckii* subsp. *sunkii* VG 1 (pH 3.42), *L. delbrueckii* subsp. *lactis* VG 2 (pH 3.41) and MK 13-1 (pH 3.54), *L. rhamnosus* NA 1-8 (pH 3.60), and *L. paracasei* AV 2-1 (pH 3.44) exhibited well-expressed antioxidant capacity. At 24 h the discoloured zones appear with more diffused boundaries. Two of the tested strains *L. delbrueckii* subsp. *sunkii* VG 1 and *L. paracasei* AV 2-1 showed the highest antioxidant capacity among the tested strains. Followed by *L. fermentum* TC 3-11 and NA 2-2, *L. delbrueckii* subsp. *lactis* VG 2 and MK 13-1, and *L. rhamnosus* NA 1-8. These strains also showed high antioxidant capacity. The CFSs from *L. fermentum* N 2 (pH 3.99) and N 4-5 (pH 4.00), *W. confusa* AG 2-6 (pH 3.89) and NN 1 (pH 3.92), and *L. curvatus* KG 12-1 (pH 3.90) exhibited lower antioxidative properties throughout the experiment. It can be noted that between 30 min and 4 h an even increase in the halo zone diameter is observed.

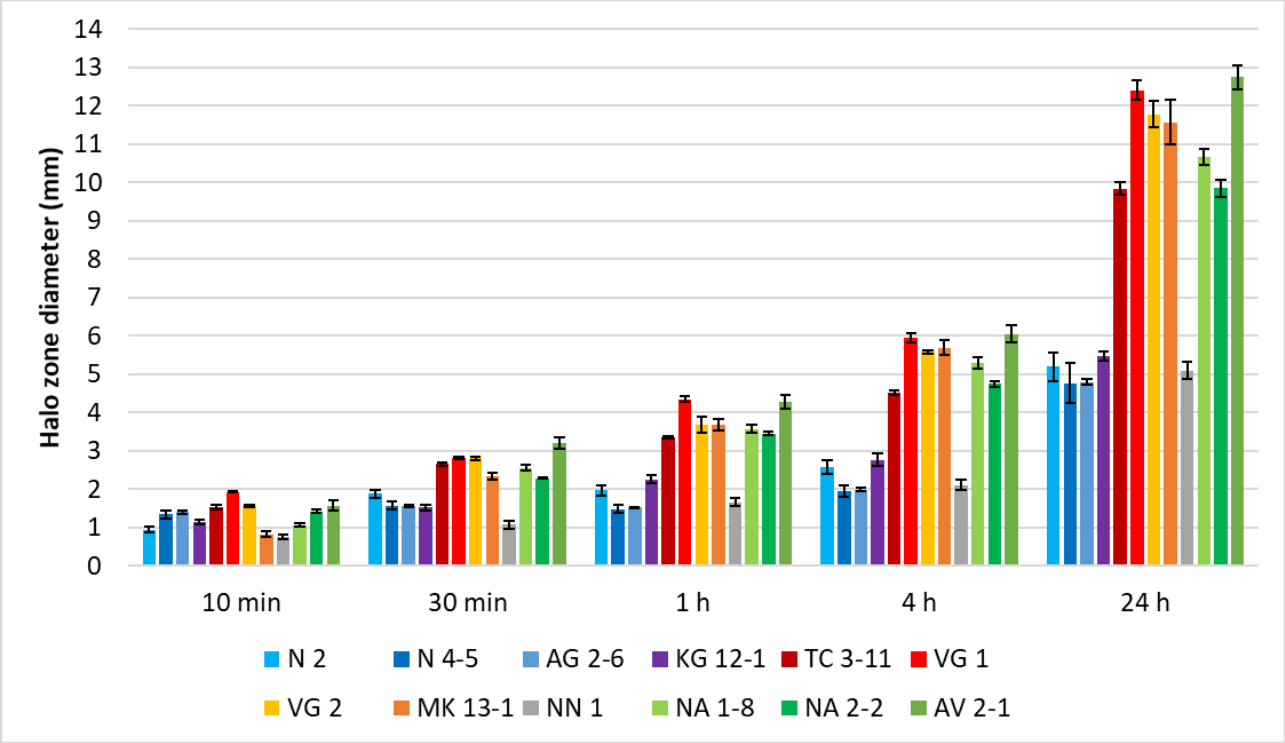

Figure 8 – Total antioxidant capacity of the tested LAB strains. Values are expressed as mean ± SD. Strains: *L. fermentum* N 2, *L. fermentum* N 4-5, *W. confusa* AG 2-6, *L. curvatus* KG 12-1, *L. fermentum* TC 3-11, *L. delbrueckii* subsp. *sunkii* VG 1, *L. delbrueckii* subsp. *lactis* VG 2, *L. delbrueckii* subsp. *lactis* MK 13-1, *W. confusa* NN 1, *L. rhamnosus* NA 1-8, *L. fermentum* NA 2-2, and *L. paracasei* AV 2-1.

Supernatants naturally have an acidic pH due to the produced organic acids from LAB, like lactic and acetic acid. In the antioxidant capacity assay, it can be acknowledged that the higher the acidity is, the more antioxidant activity the CFS possess. The redox reaction between the CFSs and $KMnO_4$ is quantitative, and the diameter of each discoloured zone is equivalent to the quantity of the antioxidant. Hanchi et al. have assessed that the newly LAB-adapted $KMnO_4$-agar antioxidant method is linear, accurate and repeatable at a time range between 30 min and 4 h and suitable for assaying LAB CFSs (Hanchi et al., 2022). As the assay gives data for the total antioxidant capacity, a more complex approach involving the ability of the tested LAB strains to reduce oxidative stress using free radicals in DPPH, ABTS, or Fenton reaction radical scavenging activity assays can give a better understanding of their antioxidant activity. The performed $KMnO_4$ agar assay serves as preliminary screening for antioxidant capacity, and more detailed analyses for evaluation of the antioxidant activity will be included in subsequent studies of the LAB strains.

7. Antagonistic interactions of the tested LAB strains against test-pathogens, including oral test-pathogens

7.1. Antimicrobial activity screening against test-pathogens and bioinformatics analysys for bacteriocin producing genes

Probiotics targeting oral healthcare can successfully compete with pathogenic species and support the growth of beneficial bacteria, so they can positively contribute mainly to the prevention of oral diseases as well as their therapy (Tekce et al., 2015; Nadkerny et al., 2015; Sabatini et al., 2017; Keller et al., 2018; Grusovin et al., 2020). A significant number of bacterial and yeast species can exhibit pathogenic effects and colonize different organs in the human body, including the oral cavity (Parahitiyawa et al., 2010; Han, 2015; Proença et al., 2017). Since pathogenic microorganisms can survive even in unfavourable conditions and spread relatively easily, it is important to study the possibility of LAB to inhibit their growth.

A complex approach was used in order to determine the antimicrobial interactions of the tested LAB strains, including antimicrobial activity of cell free supernatants (CFSs) against Gram+ and Gram- test-pathogens, antagonistic activity against two oral test-pathogens, as well as adhesion interactions with them.

All of the isolated strains were analyzed for an antimicrobial capability against the selected oral test pathogens *S. mutans* and *C. albicans*, and against gastrointestinal and skin test pathogens that can also colonize the oral cavity, including *B. subtilis, B. cereus, E. coli, S. aureus, S. epidermidis, Ps. aeruginosa,* and *P. acnes* by the agar well diffusion method. The results show the presence of antimicrobial activity with the native CFS.

Halo zones against *E. coli* were observed by all of the strains, except *L. curvatus* KG 12-1, and most of the strains expressed inhibitory activity against *B. subtilis* and *B. cereus*. *W. confusa* AG 2-6 and NN 1, and *L. curvatus* KG 12-1 did not show an inhibition against the two *Bacillus* test pathogens, also no inhibition was observed from *L. paracasei* AV 2-1 against *B. subtilis*, and from *L. rhamnosus* NA 1-8 or *L. fermentum* NA 2-2 against *B. cereus*. Against *Ps. Aeruginosa* the strains *L. fermentum* TC 3-11, *L. delbrueckii* subsp. *sunkii* VG 1, *L. delbrueckii* subsp. *lactis* VG 2 and MK 13-1, and *L. fermentum* NA 2-2 exhibited an inhibition. Three of the studied strains *L. delbrueckii* subsp. *sunkii* VG 1, *L. delbrueckii* subsp. *lactis* VG 2, and MK 13-1 showed an inhibition against *S. aureus*. Against *S. epidermidis, P. acnes,* and *S. mutans, W. confusa* AG 2-6 and NN 1 expressed inhibition activity. No antimicrobial activity was observed from the native CFS from the studied strains against C. albicans (Table 8).

Table 8 – Antimicrobial activity of the tested LAB strains (native CFSs) against test-pathogens. Strains: *L. fermentum* N 2, *L. fermentum* N 4-5, *W. confusa* AG 2-6, *L. curvatus* KG 12-1, *L. fermentum* TC 3-11, *L. delbrueckii* subsp. *sunkii* VG 1, *L. delbrueckii* subsp. *lactis* VG 2, *L. delbrueckii* subsp. *lactis* MK 13-1, *W. confusa* NN 1, *L. rhamnosus* NA 1-8, *L. fermentum* NA 2-2, and *L. paracasei* AV 2-1.

Strain	pH of the native CFS	Halo zone diameter (мм)								
		E. coli	*B. subtilis*	*B. cereus*	*S. aureus*	*S. epidermidis*	*P. aeruginosa*	*P. acnes*	*S. mutans*	*C. albicans*
N 2	3.99	13.0 ± 0.5	12.0 ± 0.3	12.0 ± 0.5	NZ	NZ	NZ	NZ	NZ	NZ
N 4-5	4.00	13.5 ± 0.5	11.0 ± 0.2	11.0 ± 0.3	NZ	NZ	NZ	NZ	NZ	NZ
AG 2-6	3.89	12.0 ± 0.2	NZ	NZ	NZ	13.0 ± 0.1	NZ	12.0 ± 0.2	12.0 ± 0.3	NZ
KG 12-1	3.90	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
TC 3-11	3.72	13.0 ± 0.5	11.0 ± 0.3	12.0 ± 1.0	NZ	NZ	11.0 ± 0.1	NZ	NZ	NZ
VG 1	3.42	17.0 ± 0.5	14.6 ± 0.6	14.6 ± 0.6	13.5 ± 0.5	NZ	11.0 ± 0.2	NZ	NZ	NZ
VG 2	3.41	17.6 ± 0.4	15.3 ± 0.2	14.0 ± 0.1	13.0 ± 0.1	NZ	11.0 ± 0.2	NZ	NZ	NZ
MK 13-1	3.54	17,0 ± 0,5	15.5 ± 0.5	14.6 ± 0.4	12.0 ± 0.1	NZ	11.0 ± 0.2	NZ	NZ	NZ
NN 1	3.92	13.0 ± 0.2	NZ	NZ	NZ	12.0 ± 0.1	NZ	12.0 ± 0.1	13.0 ± 0.2	NZ
NA 1-8	3.60	14.0 ± 0.1	13.2 ± 0.3	NZ	NZ	NZ	NZ	NZ	NZ	NZ
NA 2-2	3.92	13.0 ± 0.3	12.5 ± 0.5	NZ	NZ	NZ	12.0 ± 1.0	NZ	NZ	NZ
AV 2-1	3.44	13.0 ± 0.1	NZ	12.0 ± 0.1	NZ	NZ	NZ	NZ	NZ	NZ

NZ – no inhibition zone; values are presented as means ± SD (n = 3).

In contrast, the neutralized CFS from all of the newly isolated LAB strains did not show activity against the used test pathogens, which is related to the standard cultivation conditions of the strains without any specific metabolite inducing factors.

The results from the antimicrobial activity of the different tested strains against different test pathogens show specificity, but it was only exhibited from the native CFSs. The production of organic acids is one of the most important mechanisms of the antimicrobial activity of LAB, by which they act as antagonists of many pathogenic species. It can be assumed that the exhibited antimicrobial activity of the native CFS is mainly due to the production of organic acids from LAB–lactic acid or lactic and acetic acids (Gao et al., 2019). A study by Ren et al. with *Lactiplantibacillus pentosus*, *L. plantarum,* and *L. paracasei* showed antibacterial activity against *E. coli*, *B. cereus*, *S. aureus,* and *S. enterica* (Ren et al., 2018). Another study by Matevosyan et al. evaluated the antibacterial characteristics of LAB strains, including *L. rhamnosus*, resulting in the inhibition of the pathogens *E. coli*, *Ps. aeruginosa*, *S. aureus*, *Salmonella typhimurium*, *Bacillus mesentericus,* and *M. luteus* (Matevosyan et al., 2019). Against *S. mutans*, antibacterial activity is widely reported (Vuotto et al., 2014). According to Sookkhee et al., *L. paracasei* subsp. *paracasei* and *L. rhamnosus* expressed a high antagonizing capacity against some of the significant oral pathogens *S. mutans*, *S. sanguinis*, and *P. gingivalis* (Sookkhee et al., 2001). Strains of *L. salivarius*, *L. delbrueckii* subsp. *lactis и L. fermentum*, tudied by Strahinic et al., expressed antagonistic activity against *S. mutans* and *Streptococcus pneumoniae* (Strahinic et al., 2007).

For opportunistic oral pathogens such as *C. albicans*, an antagonistic effect is rarely observed and research shows that the inhibitory capability of LAB against *Candida* representatives is not a species-specific trait and depends on the origin of the LAB isolates (Itapary dos Santos et al., 2019). Many studies relate to the inhibitory activity to LAB isolates of a human origin. Other studies report that many used LAB did not exhibit an inhibitory activity against *Candida* (Denkova et al., 2013; Ariningsih et al., 2017; Itapary dos Santos et al., 2019; Liao et al., 2019). Many researchers continue to study microorganisms with activity against *C. albicans* and identify and isolate metabolites with inhibitory effects, since this pathogen causes diseases not only in the oral cavity, but in other organs in the human body.

LAB can synthesize other substances with antimicrobial activity, such as bacteriocins. Synthesis of such substances can be induced by different factors for strains that possess the corresponding genetic determinants. An *in silico* analysis of the whole genome sequences was held and the results show that some of the tested strains possess mechanisms for bacteriocin production (Table 9).

The obtained results are a prerequisite for further evaluationof the antagonistic activities of the tested LAB strains. Studying the antagonistic activity is on of the main factors for evaluating the direct interactions of potential probiotic LAB against different pathogens. Due to their wide prevalence in the environment and their inclusion in the composition of many products, LAB exhibit a diverse spectrum of inhibitory activities, as well as the possibility for biocontrol of different diseases. The antagonistic interactions include activities, such as the ability to adhere and inhibit pathogen adhesion to the mucosal tissue; aggregation

capabilities, including co-aggregation; production of antimicrobial metabolites, including bacteriocins (Choi et al., 2018; Leska et al., 2022).

Table 9 – Detected bacteriocin producing mechanisms for the tested strains.

Bacteriocins	Strains
Blp family class II bacteriocin	*L. curvatus* KG 12-1; *L. paracasei* AV 2-1
leucocin A/sakacin P family class II bacteriocin	*L. curvatus* KG 12-1
class IIb bacteriocin, lactobin A/cerein 7B family	*L. rhamnosus* NA 1-8
helveticin J family class III bacteriocin	*L. delbrueckii* subsp. *sunkii* VG 1; *L. delbrueckii* subsp. *lactis* VG 2; *L. delbrueckii* subsp. *lactis* MK 13-1

7.2. Antagonistic activity against oral test-pathogens by co-cultivation

In order to evaluate their competitiveness in the environment of the oral cavity, it is important for the direct interactions of LAB with oral pathogens to be studied. The antagonistic activity is evaluated by quantitative and qualitative methods. From the spot assay, inhibition zones were reported from all of the tested LAB strains against *S. mutans*. Against *C. albicans*, inhibition zones were observed from eight of the studied strains: *L. fermentum* N2, N 4-5, TC 3-11 and NA 2-2, *L. delbrueckii* subsp. *lactis* VG 2, *W. confusa* NN 1, *L. rhamnosus* 1-8, and *L. paracasei* AV 2-1 (Table 10).

Table 10 – Inhibitory activity of the tested LAB strains against *S. mutans* and *C. albicans*.

Strain	*S. mutans*	*C. albicans*
L. fermentum N 2	+	+
L. fermentum N 4-5	+	+
W. confusa AG 2-6	+	-
L. curvatus KG 12-1	+	-
L. fermentum TC 3-11	+	+
L. delbrueckii subsp. *sunkii* VG 1	+	-
L. delbrueckii subsp. *lactis* VG 2	+	+
L. delbrueckii subsp. *lactis* MK 13-1	+	-
W. confusa NN 1	+	+
L. rhamnosus NA 1-8	+	+
L. fermentum NA 2-2	+	+
L. paracasei AV 2-1	+	+

The results are a primary screening that the tested strains exhibit antagonistic properties against the selected oral pathogens. The direct co-culturing assay was performed to quantitatively evaluate the antagonistic activity of the tested LAB strains against the selected pathogens (Figure 9).

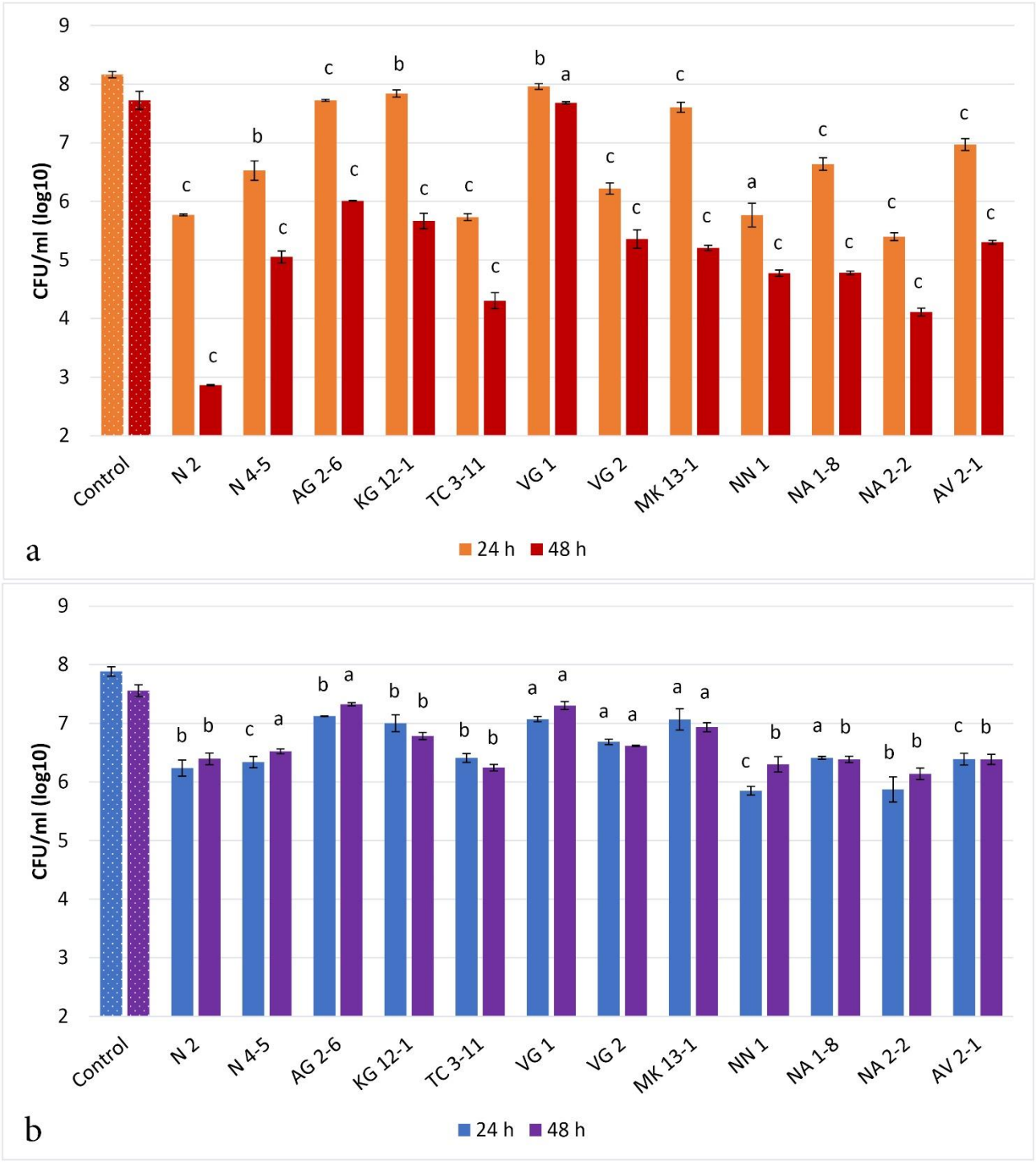

Figure 9 – Antagonistic activity of the tested LAB strains in direct co-cultivation with S. mutans (**a**) and C. albicans (**b**). Values are expressed as mean ± SD. Statistical analysis was performed by Student's t-test: a – nonsignificant ($p > 0.05$); b and c – significant ($p < 0.05$ and $p < 0.01$, respectively). Strains: *L. fermentum* N 2, *L. fermentum* N 4-5, *W. confusa* AG 2-6, *L. curvatus* KG 12-1, *L. fermentum* TC 3-11, *L. delbrueckii* subsp. *sunkii* VG 1, *L. delbrueckii* subsp. *lactis* VG 2, *L. delbrueckii* subsp. *lactis* MK 13-1, *W. confusa* NN 1, *L. rhamnosus* NA 1-8, *L. fermentum* NA 2-2, and *L. paracasei* AV 2-1.

The results from the co-culturing assay showed that at 24 h *L. fermentum* N2, N 4-5, TC 3-11 and NA 2-2, *L. delbrueckii* subsp. *lactis* VG 2, *W. confusa* NN 1, *L. rhamnosus* NA 1-8, and *L. paracasei* AV 2-1 exhibit antagonistic activity against *S. mutans* by decreasing the viability of the pathogen by 1–3 logs. At 48 h antagonistic activity was reported from more of the tested LAB strains – *L. fermentum* N2, N 4-5, TC 3-11 and NA 2-2, *L. delbrueckii* subsp. *lactis* VG 2 and MK 13-1, *W. confusa* AG 2-6 and NN 1, *L. rhamnosus* 1-8, and *L. paracasei* AV 2-1 exhibited a decrease in pathogen live cell number by 2–5 logs. Only one strain *L. delbrueckii* subsp. *sunkii* VG 1 had no activity against *S. mutans* (Figure 22a). All of the tested LAB strains showed slightly expressed antagonistic activity against *C. albicans* by decreasing the viability of the pathogen by 1–2 logs until 48 h (Figure 22b).

Both *S. mutans* and *C. albicans* significantly contribute to the pathogenesis of caries, candidiasis, and periodontitis infections (Chevalier et al., 2018; Morrison et al., 2023). LAB also play a crucial role in the oral ecosystem by contributing to oral health (Badet and Thebaud, 2008). The obtained data from the co-culturing assay shows significant inhibition of *S. mutans* from most of the tested LAB strains. Most importantly, *L. fermentum* N 2, TC 3-11 and NA 2-2, and *W. confusa* NN 1 exhibited the highest impact on the cell density of the pathogen throughout the co-cultivation.

For *C. albicans*, it can be observed that the same eight strains that showed antagonistic activity in the spot analysis exhibited better activity in the co-cultivation assay. On the other hand, the yeast pathogen proves its opportunistic status (Lopes and Lionakis, 2022) as only two of the LAB strains, *L. fermentum* NA 2-2 and *W. confusa* NN 1, showed up to 2 logs of inhibition at 24 h.

In the assay performed by Denkova et al., the authors used the *L. acidophilus* strain against *E. coli*, *Staphylococcus* and *Salmonella* sp., where the LAB strain reduced pathogenic cell density by 1–2 logs until 24 h and 4–8 logs until 48 h of the co-culturing (Denkova et al., 2022). In a study by Chen et al., *L. fermentum* and *L. salivarius* showed definite inhibition of oral cariogenic and periodontal bacteria, including *S. mutans*, *S. sanguinis* and *P. gingivalis* (Chen et al., 2012). In another study, Mann et al. evaluated *Lactobacillus gasseri*, which reduced the cell density of oral streptococci, *Porphyromonas* sp. and *F. nucleatum* as low as 10^5 CFU/mL (Mann et al., 2021b). In a previous study by Denkova et al., the authors evaluated the antagonistic activity of *L. acidophilus* and *L. delbrueckii* subsp. *bulgaricus*, which reduced the cell density of *C. albicans* by 2 logs at 24 h and 2 to 3 logs at 48 h of co-culturing (Denkova et al., 2013). A study by Vazquez-Munoz et al. assessed a *Lactobacillus johnsonii* strain that showed inhibition of *C. albicans* by 10.1% in a 1:1 cell ratio co-cultivation for 24 h. (Vazquez-Munoz et al., 2022).

It is important to note that eleven of the LAB strains included in our study possess expressed antagonistic activity against S. mutans, which has a statistically significant decrease in the cell density of the pathogen. The obtained data from the co-cultivation assay on the tested LAB strains in our study support and substantially add to the results in the studies of other authors cited above. The scientific publications are limited to research on LAB being evaluated in co-cultivation assays with cariogenic and periodontal pathogens. Research on *C. albicans*, however, is extensive due to the opportunistic nature of the

pathogen and its widespread human infection niches (Lopes and Lionakis, 2022). As a novelty, our research evaluated *L. curvatus*, *L. rhamnosus*, *L. paracasei*, and *W. confusa* strains, previously not studied in co-culturing techniques with oral pathogenic microorganisms. Our research provides confirmation of the direct interactions of the LAB included in our study with oral pathogenic microorganisms, especially with *S. mutans*, regarding their antagonistic properties in the composition of the oral microbiota.

7.3. Co-aggregation capability

Co-aggregation represents the intercellular adhesion properties of different microorganisms (Khemaleelakul et al., 2006). All subjected to co-aggregation LAB strains indicated co-aggregative properties with the selected oral test pathogens. With *S. mutans*, all tested LAB strains exhibited co-aggregation in the range of 9.22–19.67% higher than the autoaggregation of the test pathogen, measured at 6.21%. The highest co-aggregation was observed from *L. fermentum* NA 2-2 and *L. rhamnosus* NA 1-8, measured at 19.67% and 18.78%, respectively. With *C. albicans*, all tested LAB strains exhibited co-aggregation in the range of 9.83–27.97%, lower than the autoaggregation of the test pathogen, measured at 29.41%. *L. delbrueckii* subsp. *lactis* VG 2 and MK 13-1 showed the highest co-aggregation with the test pathogen at 27.97% and 26.76%, respectively (Figure 10).

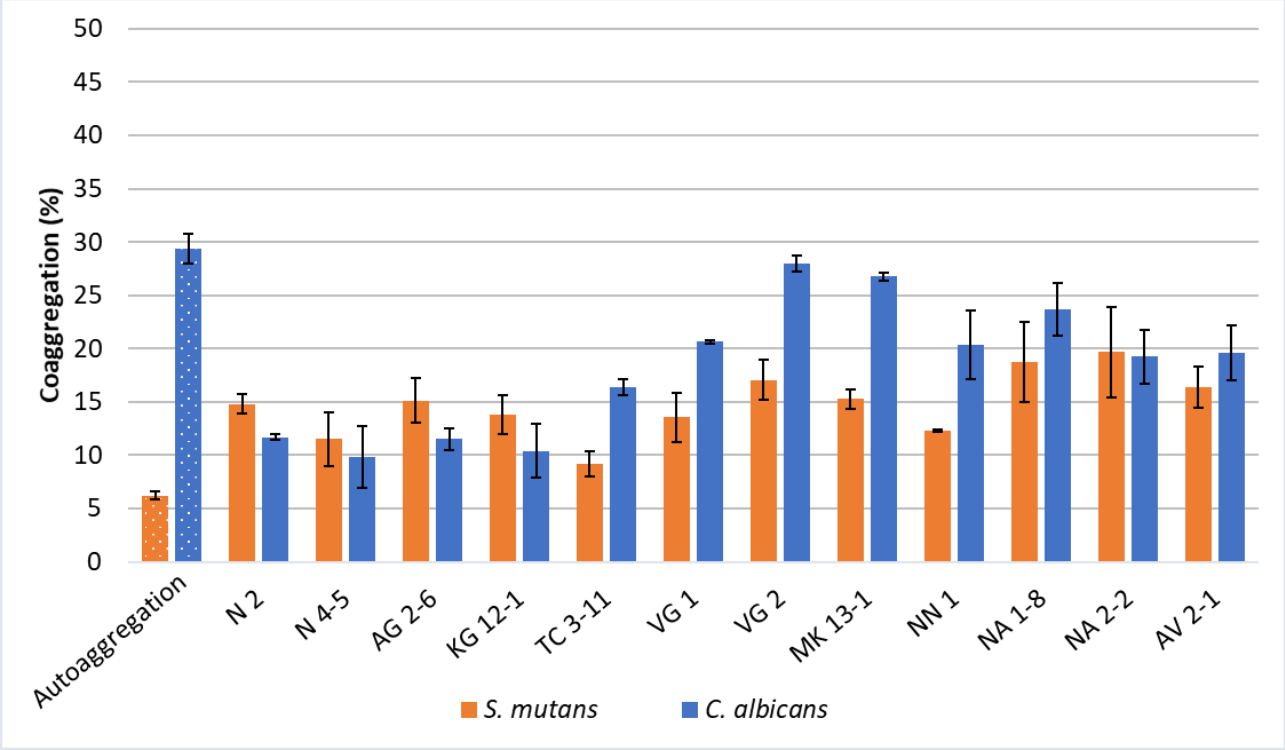

Figure 10 – Co-aggregation of the tested LAB strains with *S. mutans* and *C. albicans*. Values are expressed as mean ± SD. Statistical analysis was performed by One-way ANOVA: a – nonsignificant ($p > 0.05$); c – significant ($p < 0.01$). Strains: *L. fermentum* N 2, *L. fermentum* N 4-5, *W. confusa* AG 2-6, *L. curvatus* KG 12-1, *L. fermentum* TC 3-11, *L. delbrueckii* subsp. *sunkii* VG 1, *L. delbrueckii* subsp. *lactis* VG 2, *L. delbrueckii* subsp. *lactis* MK 13-1, *W. confusa* NN 1, *L. rhamnosus* NA 1-8, *L. fermentum* NA 2-2 and *L. paracasei* AV 2-1.

The determination of the potential aggregative properties of LAB is influenced by internal and environmental factors (Goh and Klaenhammer, 2010). o-aggregation has been observed between microbial species in the oral microbiome's composition. It is suggested that LAB, which can co-aggregate with oral pathogens, may exert an important host defence mechanism against infection development (Rickard et al., 2003). From the obtained data, all tested LAB strains in our study possess the property to co-aggregate with *S. mutans*, and most of them showed well-expressed co-aggregation with *C. albicans*. Statistically, no significant difference was reported for the tested LAB strains in co-aggregation with *S. mutans*. However, statistically significant differences among the tested strains were reported in co-aggregation with *C. albicans*. This shows specificity in their co-aggregative properties with the yeast pathogen and can be treated as a strain-specific property. It can be observed that three of the tested LAB strains possess well-expressed co-aggregation with *C. albicans*: *L. delbrueckii* subsp. *lactis* VG 2 and MK 13-1, and *L. rhamnosus* NA 1-8. Strain specificity was reported by other authors as well. LAB strains studied by Ciandrini et al., including*L. paracasei* and *L. rhamnosus*, showed co-aggregative properties with *S. mutans* in the range of 6.32–20.93 % (Ciandrini et al., 2017). In a study by Lai et al., five LAB strains were evaluated, and co-aggregation with *S. mutans* was reported between 15.93 % и 62.25 % (Lai et al., 2021). In a study by Aarti et al., the evaluated *L. pentosus* strain exhibited 37.1 % co-aggregative properties with *C. albicans* (Aarti et al., 2018). Malfa et al. used a multistrain formulation of LAB strains, *L. rhamnosus* strain included, and observed high co-aggregation capability with *C. albicans* (Malfa et al., 2023).

7.4. *In vitro* evaluation of antibiofilm activity against oral test-pathogens

The ability of oral LAB to inhibit biofilms of pathogens in the oral cavity is essential for their application as oral probiotics. From the held antibiofilm assay, nine of the tested LAB strains exhibited inhibition of biofilm formation by *S. mutans*, and all strains inhibited biofilm formation of *C. albicans* (Figure 11). Against *S. mutans*, eight of the tested strains exhibited definite biofilm inhibitory activity, and only three did not exhibit inhibition in the conditions of the *in vitro* analysis. The strain *L. fermentum* NA 2-2 showed excellent pathogen biofilm inhibition properties by 100%. Following this result, *L. fermentum* N 2, N 4-5 and TC 3-11, *W. confusa* AG 2-6 and NN 1, *L. curvatus* KG 12-1, and *L. rhamnosus* NA 1-8 also expressed high biofilm inhibition properties, measured above 79%. From the tested LAB strains, *L. delbrueckii* subsp. *lactis* VG 2 showed to possess the lowest biofilm inhibition. For *L. delbrueckii* subsp. *sunkii* VG 1, *L. delbrueckii* subsp. *lactis* MK 13-1 and *L. paracasei* AV 2-1, no biofilm inhibition properties were observed in this *in vitro* analysis. Against *C. albicans*, all of the tested LAB strains possess antibiofilm activity. The strain *L. fermentum* NA 2-2 exhibited the highest biofilm inhibition, above 81%. This strain, along with *L. fermentum* N 2, N 4-5 and TC 3-11, and *W. confusa* AG 2-6, showed to inhibit biofilm formation that exceeds 60%

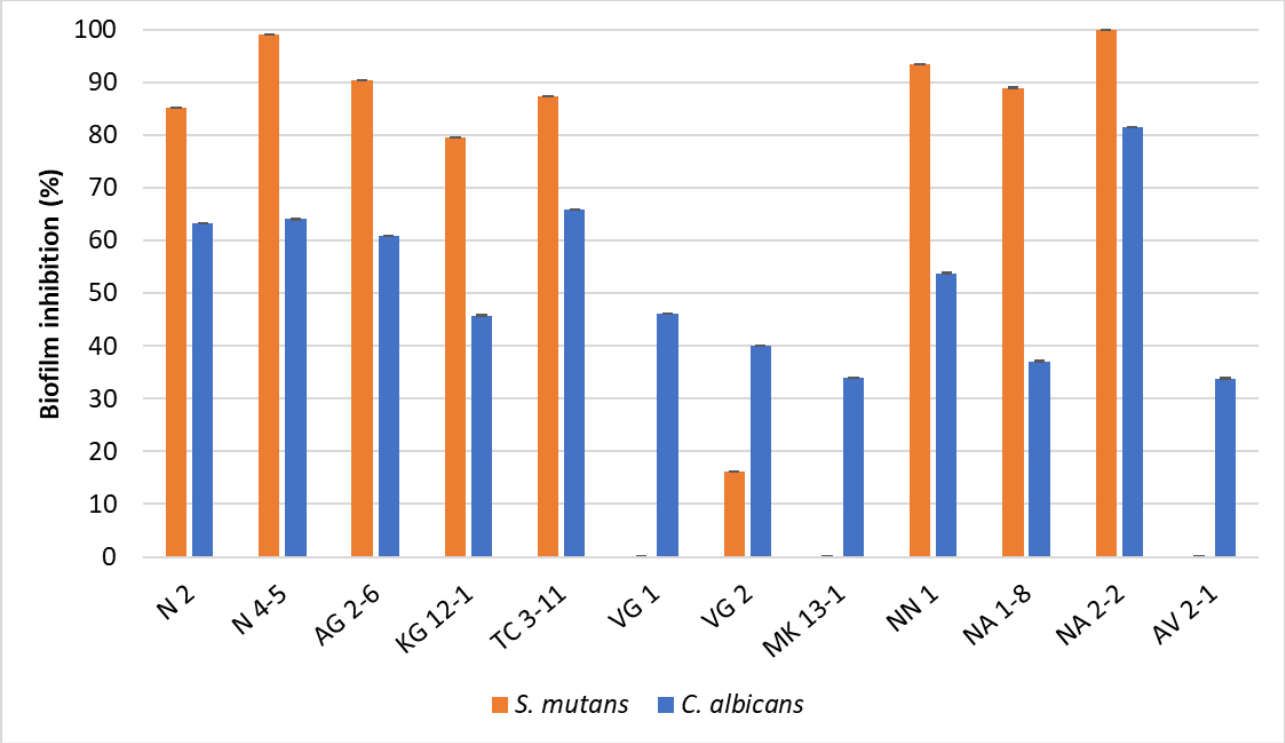

Figure 11 – Percent of biofilm inhibition by the tested LAB strains against *S. mutans* and *C. albicans*. Values are expressed as mean ± SD. Statistical analysis was performed by One-way ANOVA ($p < 0.01$). Strains: *L. fermentum* N 2, *L. fermentum* N 4-5, *W. confusa* AG 2-6, *L. curvatus* KG 12-1, *L. fermentum* TC 3-11, *L. delbrueckii* subsp. *sunkii* VG 1, *L. delbrueckii* subsp. *lactis* VG 2, *L. delbrueckii* subsp. *lactis* MK 13-1, *W. confusa* NN 1, *L. rhamnosus* NA 1-8, *L. fermentum* NA 2-2 and *L. paracasei* AV 2-1.

S. mutans produces cell-wall anchored proteins that facilitate binding to *C. albicans*, which successively assist streptococcal colonization and further caries development from the formed biofilm (Bamford et al., 2009; Matsuda et al., 2018; Morrison et al., 2023). In the antibiofilm assay, *L. fermentum* NA 2-2 exhibited the highest biofilm inhibition against both pathogens, possessing the capacity to prevent the accumulation of pathogenic biofilms *in vitro*. From the results, it can be suggested that antibiofilm activity against the selected oral pathogens could be a species-related activity. All four *L. fermentum* strains biofilm inhibition was high against both pathogens. *W. confusa* AG 2-6 and NN 1 showed similar percentages of inhibition against the test pathogens. The similarity in biofilm inhibition against *C. albicans* was also shown by the tested *L. delbrueckii* subsp. *sunkii* VG 1, and *L. delbrueckii* subsp. *lactis* VG 2 and MK 13-1 strains.

In a study by Patel et al., the authors evaluated glycolipid biosurfactant derived from a newly isolated *L. rhamnosus* strain which was effective in inhibiting biofilms of *B. subtilis*, *E. coli*, *P. aeruginosa,* and *S. aureus* (Patel et al., 2021). In the study by Jha et al., the six tested LAB strains showed biofilm inhibition properties against *S. mutans* in the 2.09–15.07 % range (Jha et al., 2022). Wasfi et al. studied four *Lactobacillus* sp. strains, which showed antibiofilm activity against *S. mutans* from 24.7 to 47 % (Wasfi et al., 2018).

The constant presence of LAB strains can potentially increase their intervention with pathogens. In that matter, LAB species normally found in the composition of the oral

microbiome possess higher antibiofilm activity, as seen from the results in our study. Wu et al. evaluated sixty-four strains of *L. salivarius* from human saliva, inhibiting biofilm formation of *S. mutans* up to 69 % (Wu et al., 2015). Krzyściak et al. showed that *L. salivarius* reduced the biomass of both mono-species biofilms of *S. mutans* and *C. albicans,* and multispecies biofilm (Krzyściak et al., 2017). Rossoni et al. evaluated *L. fermentum* 20.4, *L. paracasei* 28.4, and *L. rhamnosus* 5.2 strains, reducing the biofilm development of *C. albicans* ATCC 18804 and clinical isolates (Rossoni et al., 2018). James et al. have evaluated a multistrain LAB combinations that exerted high effectiveness against *C. albicans* biofilms (James et al., 2016). The obtained results in the antibiofilm activity on the tested LAB strains in our study show that eight of the strains possess better antibiofilm properties against *S. mutans* than the LAB strains evaluated by other authors cited above. Also, the antibiofilm activity against *C. albicans* was noticeably high, which suggests an effective influence from the LAB strains against biofilm formation from the yeast pathogen.

7.5. Correlation analysis

The results from the co-cultivation, co-aggregation and antibiofilm activity of the studied LAB strains were compared for correlation among them using Pearson's correlation. The obtained data indicate a positive correlation between the antagonistic activity of the studied strains against both test pathogens in co-cultivation and antibiofilm activity. A positive correlation was reported between the co-aggregation and antibiofilm activity assays of the LAB strains against *C. albicans*. No positive correlation was determined between the co-aggregation ability and the antibiofilm activity of the studied strains against *S. mutans*. Based on the established correlations, it can be supposed that the main antagonistic mechanisms of the LAB strains against *S. mutans* are related to their metabolic activity. The established antagonistic activity against *C. albicans* is more likely determined due to the direct physical exclusion of this opportunistic pathogen.

8. Survival ability of the tested LAB strains after freeze-drying and storage

The determination of the technological application of LAB strains with probiotic potential also includes evaluation of their stability and viability in different technological processes (Fonseca et al., 2015). It is important to select optimal conditions for freeze-drying and lyoprotective media composition in order for the process to be successfully carried through and minimize the negative effects over the freeze-dried bacterial cells, including inactivation of membrane lipids and reduction of structural changes of sensitive proteins (Nowak and Jakubczyk, 2020). The stability of LAB during the subsequent storage is also important. Probiotic bacteria should be able to maintain high cell density of viable cells in order to be added in the composition of probiotic or other products (Fenster et al., 2019; Terpou et al., 2019).

Two lyoprotective media have been used during the freeze-drying and the process was performed by aseptically loading the vials in the freeze-dryer, monitoring the parameters of and closing of the vials under vacuum at the end (Figure 12).

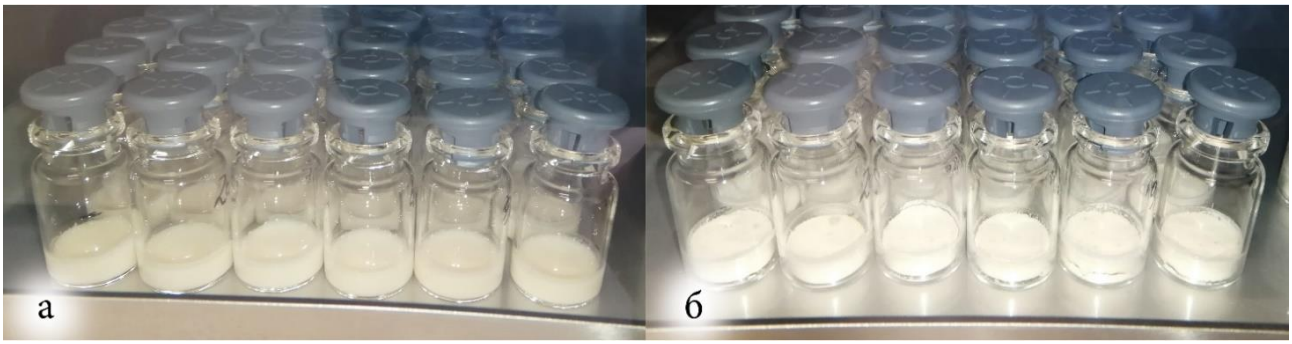

Figure 12 – Prepared samples of the tested LAB strains in the used lyoprotective media before freeze-drying (**a**) and after freeze-drying (**b**).

After freeze-drying, the dried samples were analyzed for stability, as well as after storage for four and eight months at 4°C by determining the CFU. The results show that the used lyoprotective media retain stable viability of all LAB strains during the freeze-drying process (Figure 13).

In the SML lyoprotectant, *L. fermentum* N 2, *L. curvatus* KG 12-1, *W. confusa* NN 1, *L. rhamnosus* NA 1-8 and *L. paracasei* AV 2-1, retain their cell density at 10^9 CFU/mL, and *L. delbrueckii* subsp. *lactis* VG 2 and MK 13-1 at 10^7 CFU/mL after the end of the freeze-drying process. No statistically significant difference in CFU/mL is observed for *L. fermentum* NA 2-2. For *L. fermentum* N 4-5 and TC 3-11, cell density decreases to 10^9 CFU/mL, and for *W. confusa* AG 2-6 and *L. delbrueckii* subsp. *sunkii* VG 1 to 10^7 CFU/mL. In the SMT lyoprotectant, *L. fermentum* N 2, N 4-5, TC 3-11 and NA 2-2, *L. curvatus* KG 12-1, *W. confusa* NN 1, *L. rhamnosus* NA 1-8, and *L. paracasei* AV 2-1 retain their cell density at 10^9 CFU/mL, *L. delbrueckii* subsp. *lactis* VG 2 at 10^8 CFU/mL and *L. delbrueckii* subsp. *lactis* MK 13-1 at 10^7 CFU/mL after the end of the freeze-drying process. For *W. confusa* AG 2-6 and *L. delbrueckii* subsp. *sunkii* VG 1 cell density decreases to 10^7 CFU/mL. The viability decrease of the tested LAB strains after freeze-drying is less than 1 log in both lyoprotectors. Although the small differences in survival ability, the two constructed lyoprotective media ensured excellent stability of LAB during the freeze-drying process.

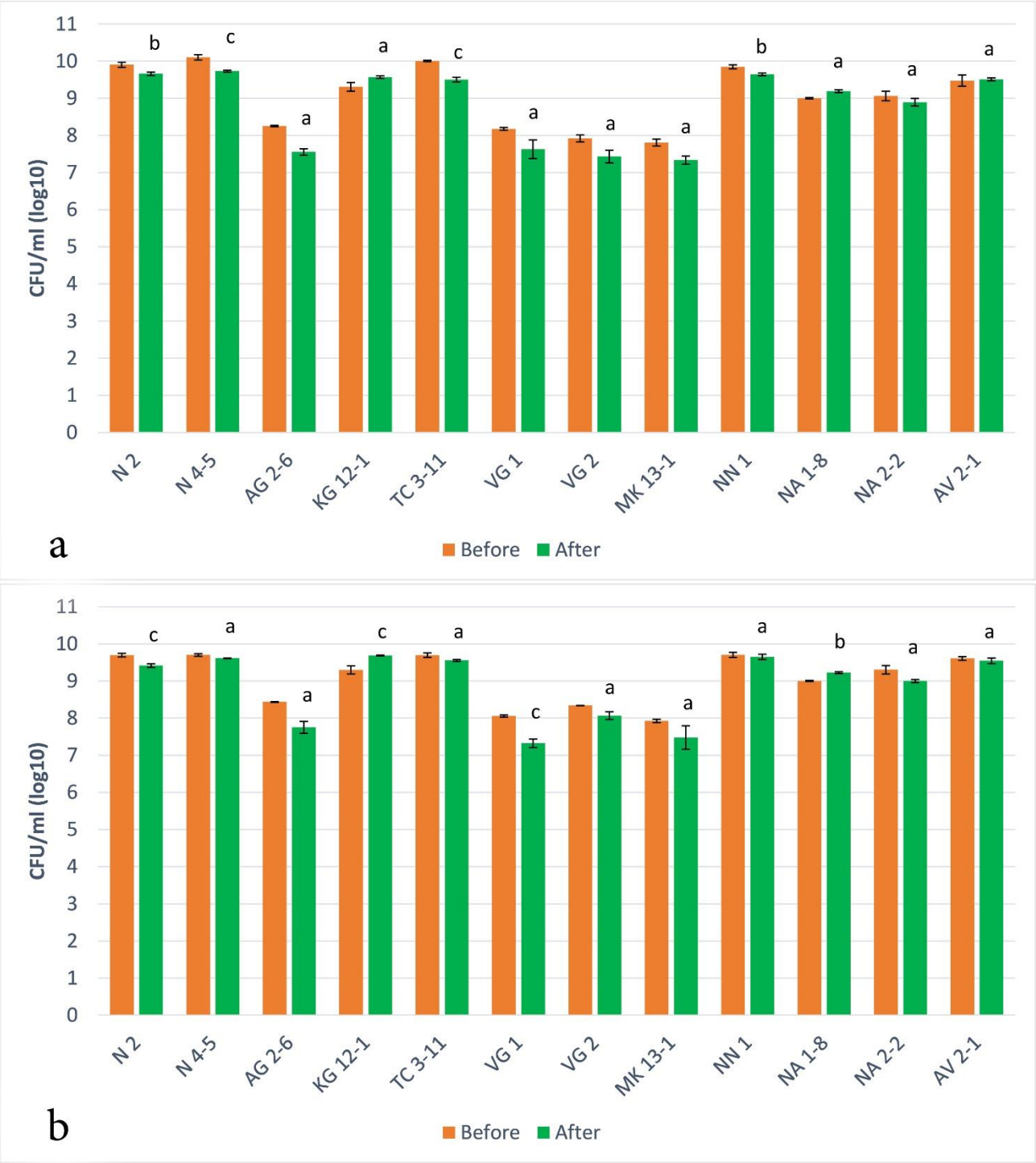

Figure 13 – Viability of the tested LAB strains after freeze-drying process in lyoprotectors SML (**a**) and SMT (**b**). Values are expressed as mean ± SD. One-way ANOVA + post-hoc Tukey test: a – $p > 0.05$; b and c – $p < 0.05$ and $p < 0.01$, respectively. Strains: *L. fermentum* N 2, *L. fermentum* N 4-5, *W. confusa* AG 2-6, *L. curvatus* KG 12-1, *L. fermentum* TC 3-11, *L. delbrueckii* subsp. *sunkii* VG 1, *L. delbrueckii* subsp. *lactis* VG 2, *L. delbrueckii* subsp. *lactis* MK 13-1, *W. confusa* NN 1, *L. rhamnosus* NA 1-8, *L. fermentum* NA 2-2 and *L. paracasei* AV 2-1.

The results from the long-term storage show that all of the tested LAB strains retain high viability after four and eight months of storage at 4°C (Figure 14.1; 14.2).

In the SML lyoprotectant, *L. fermentum* N 2, *L. curvatus* KG 12-1, *L. delbrueckii* subsp. *lactis* VG 2 and MK 13-1, *W. confusa* NN 1, *L. rhamnosus* NA 1-8 and *L. paracasei* AV 2-1 maintain their cell density unchanged for the whole period of storage. In the SMT lyoprotectant, *L. fermentum* N 2, N 4-5 and TC 3-11, *W. confusa* NN 1, *L. rhamnosus* NA 1-8 and *L. paracasei* AV 2-1 maintain their cell density unchanged for the 8-month storage. For *L. fermentum* N 2, *L. curvatus* KG 12-1, *W. confusa* NN 1, *L. rhamnosus* NA 1-8, and *L. paracasei* AV 2-1 no differences in CFU/mL are observed in both lyoprotective media, but for *L. fermentum* N 4-5, TC 3-11 and NA 2-2, *L. delbrueckii* subsp. *lactis* VG 2, and *W. confusa* AG 2-6 show better stability in the SMT lyoprotector.

In a study by Montel Mendoza et al., the authors evaluated *Lactococcus* strains and *Lactiplantibacillus plantarum* CRL 1606 in nine lyoprotective media, where two of them, comprised of only lactose and skim milk + lactose showed excellent viability preservation after freeze-drying (Montel Mendoza et al., 2014). Jalali et al. evaluated ten lyoprotectors with different ratio of skim milk, trehalose and sodium ascorbate. Freeze-drying of *Lacticaseibacillus paracasei* subsp. *tolerans* DSM 20258 and *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081 showed that the studied strains were most stable in a protector with 6 % skim milk, 8 % trehalose and 4 % sodium ascorbate (Jalali et al., 2012). Studies by Gul et al. and Ren et al. used different protective media, comprised of skim milk, lactose and sucrose for freeze-drying of *Ligilactobacillus salivarius* and *Ligilactobacillus agilis*, and *L. curvatus* N 19, respectively. The results indicated that increasing the skim milk concentration resulted in an increase of cell viability and lactose and sucrose retained cell stability after freeze-drying (Ren et al., 2019; Gul et al., 2020a).

To summarise, *L. fermentum* N 2, *W. confusa* NN 1, *L. rhamnosus* NA 1-8 and *L. paracasei* AV 2-1 show better survival ability after freeze-drying and maintain their viability better after long-term storage in both lyoprotective media. In the SML lyoprotectant, *W. confusa* NN 1, *L. rhamnosus* NA 1-8 and *L. paracasei* AV 2-1 are observed to have better stability than the other strains and in the SMT lyoprotectant, *L. fermentum* N 2, N 4-5 and TC 3-11, *W. confusa* NN 1, *L. rhamnosus* NA 1-8 and *L. paracasei* AV 2-1 were more stable. These results point that the stability of the studied LAB is better retained when trehalose is added to the lyoprotectant formulation.

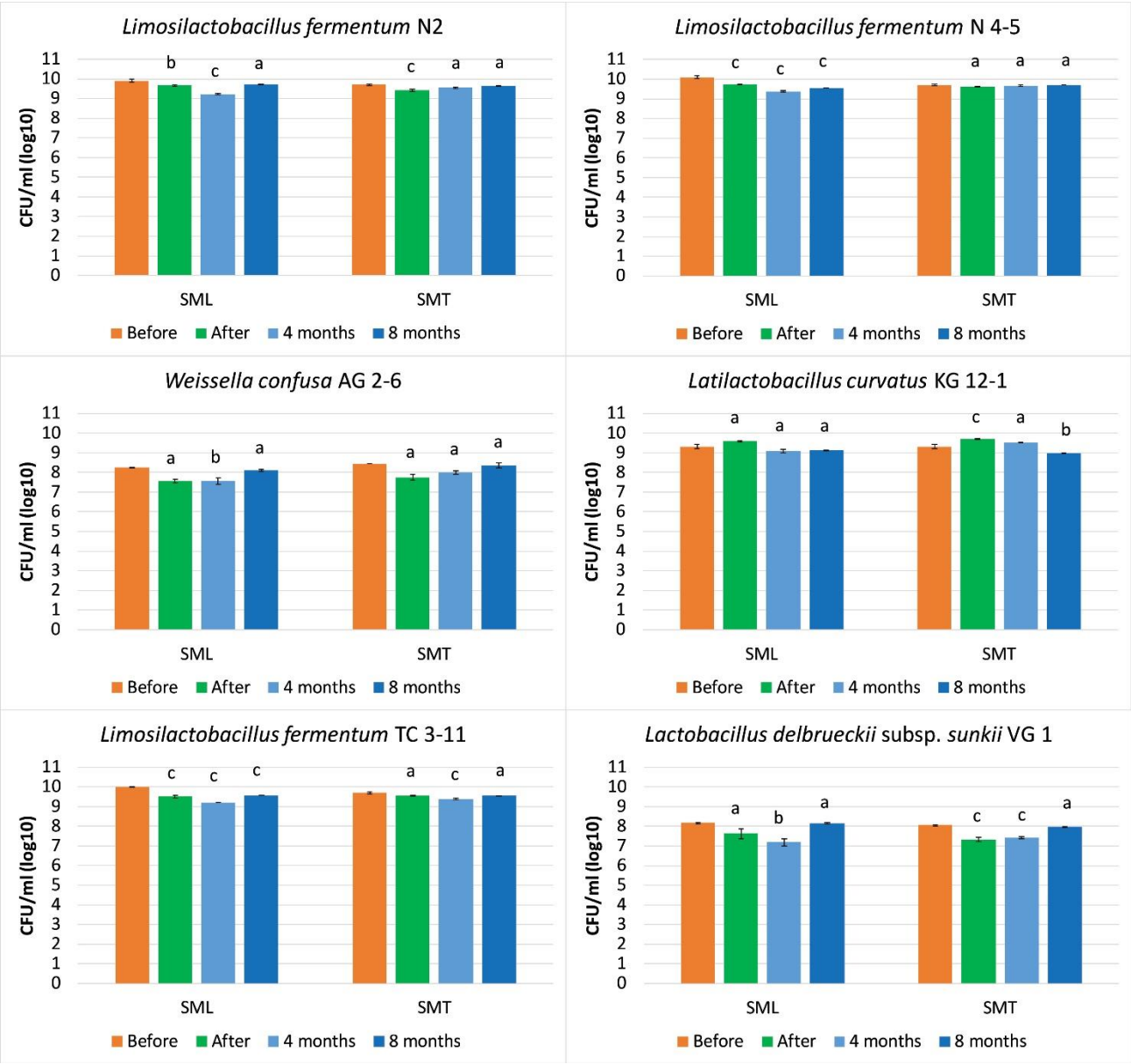

Figure 14.1 – Viability of the tested LAB strains after storage at 4°C for four and eight months in lyoprotectors SML and SMT. Values are expressed as mean ± SD. One-way ANOVA + post-hoc Tukey test: a – $p > 0.05$; b and c – $p < 0.05$ and $p < 0.01$, respectively.

Different authors reported that better survival rates were observed after long-term storage at 4°C (Shu et al., 2018; Savedboworn et al., 2019; Gul et al., 2020b). In the study by Jalali et al., the studied *L. paracasei* subsp. *tolerans* DSM 20258 and *L. delbrueckii* subsp. *bulgaricus* DSM 20081 were stored at 4°C for three months and the results showed that the strains were most stable in the same protector, comprised of 6 % skim milk, 8 % trehalose and 4 % sodium ascorbate (Jalali et al., 2012). In two studies by Gul et al., *L. curvatus* N 19 and *Levilactobacillus brevis* ED25 were subjected to six months of storage at refrigeration temperature in optimized protectant composition (20 % skim milk, 3.57 % lactose and 10 % sucrose, and 17.28 % skim milk, 2.12 % lactose, and 10 % sucrose, respectively) and

showed excellent stability with viability loss of less than 1 log (Gul et al., 2020a; Gul et al., 2020b). In a study by Sun et al., the authors reported that adding trehalose in the composition of freeze-drying solutions significantly increased the survival ability of *L. plantarum* LP105 after 240 days of storage at 4°C with viability decrease by less than 1 log (Sun et al., 2021).

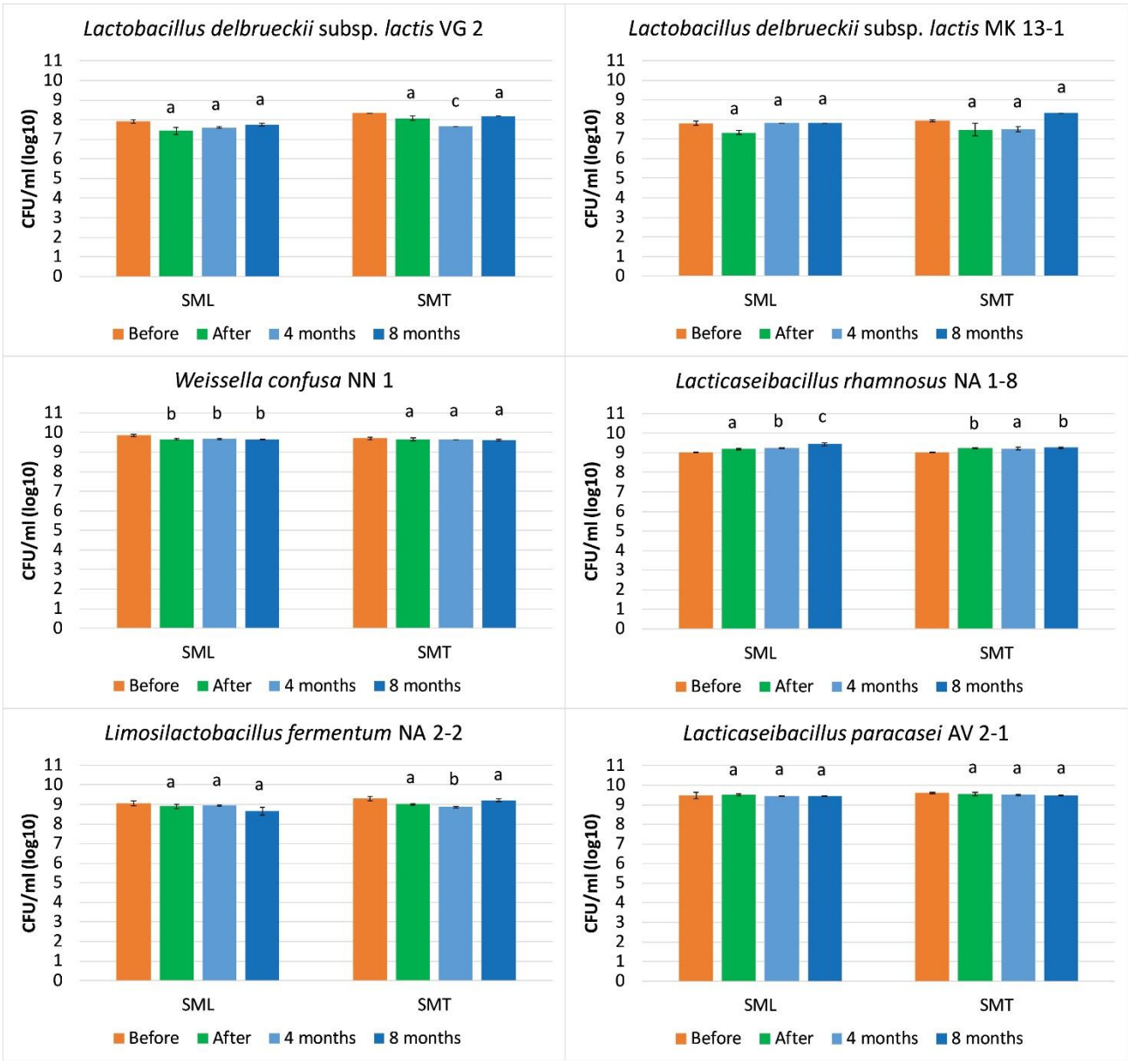

Figure 14.2 – Viability of the tested LAB strains after storage at 4°C for four and eight months in lyoprotectors SML and SMT. Values are expressed as mean ± SD. One-way ANOVA + post-hoc Tukey test: a – $p > 0.05$; b and c – $p < 0.05$ and $p < 0.01$, respectively.

9. Stability and survival ability of selected LAB strains in different model product formulations for oral health

9.1. Probiotic potential calculation and selection of strains for inclusion in the composition of model probiotic products

The probiotic potential of the tested LAB strains was determined by taking into account the analyses performed for a spectrum of functional and probiotic properties and in relation to the results obtained from these analyses by applying a scoring system to form a complex score. The probiotic potential of the tested strains with the scores for the individual parameters and the complex score as percentages are presented in Table 11 and Figure 15.

On the basis of the complex analysis, it can be reported that most of the strains possess probiotic potential above 60%, with the highest percentage determined for strain *L. fermentum* TC 3-11 – 74.3%, followed by 71.1% for *W. confusa* NN 1, 68.6 % for *L. fermentum N 2, N 4-5, NA 2-2* and *L. rhamnosus* NA 1-8, and 60.0 % for *L. delbrueckii* subsp. *lactis* VG 2 and *W. confusa* AG 2-6.

After analyzing the obtained complex score and the results for the expression of specific properties, two of the tested strains with expressed functional and probiotic characteristics were selected. According to the results from the studies, the four *L. fermentum* strains possess an expressed complex probiotic potential, and one of the main criteria for their selection is the exhibited high level of survival and potential for growth in the presence of the GIT stress factors and the oral cavity in particular. Expressed autoaggregation and mucin-binding ability were also observed for the four strains of this species. One of the main characteristics is the most expressed antagonistic activity against the two oral test-pathogens *S. mutans* and *C. albicans*. From this group of four tested strains, *L. fermentum* N 2 was selected.

The second most abundant group of studied strains from the same species are the three *L. delbrueckii* representatives, which showed broad antimicrobial activity against Gram+ and Gram- pathogens *B. cereus, B. subtilis, E. coli, Ps. aeruginosa,* and *S. aureus*. Survival in simulated oral cavity conditions as well as binding ability to mucin proteins was also determined. Of the three strains, *L. delbrueckii* subsp. *lactis* VG 2 was selected because of the exhibited self-biofilm production activity and coaggregative properties.

Table 11 – Probiotic potential of the tested LAB strains.

Probiotic characteristic	***L. fermentum* N 2**	***L. fermentum* N 4-5**	***W. confusa* AG 2-6**	***L. curvatus* KG 12-1**	***L. fermentum* TC 3-11**	***L. delbrueckii* subsp. *sunkii* VG 1**	***L. delbrueckii* subsp. *lactis* VG 2**	***L. delbrueckii* subsp. *lactis* MK 13-1**	***W. confusa* NN 1**	***L. rhamnosus* NA 1-8**	***L. fermentum* NA 2-2**	***L. paracasei* AV 2-1**
Survival ability under oral stress	2	2	1	2	2	2	2	2	2	2	2	2
Survival ability under direct impact of upper GIT compartments	2	2	1	0	2	0	1	1	1	2	2	0
Growth in the presence of oral stress factors	2	2	2	0	2	0	0	0	2	2	2	2
Growth in the presence of 0.3% bile salts stress factor	1	1	1	0	1	0	0	0	1	0	1	1
Aggregation potential	1	1	1	1	1	1	1	1	1	2	1	2
Binding to mucin	1	1	1	1	1	1	1	1	1	1	1	1
Presence of genetic determinants for adhesins and lectins	1	1	2	2	1	1	1	1	2	2	1	2
Biofilm formation	2	3	1	1	3	2	3	2	2	2	2	1
Antibiotic resistance (MAR index)	1	1	1	1	1	1	1	1	1	1	1	1
Antimicrobial activity (total of 9 test-pathogens)	3	3	4	0	4	5	5	5	4	2	3	2
Genetic determinants for bacteriocins	0	0	0	1	0	1	1	1	0	1	0	1
Antagonistic activity against oral test-pathogen *S. mutans*	2	1	1	1	2	1	1	1	2	2	2	1
Antagonistic activity against oral test-pathogen *C. albicans*	2	2	1	2	2	1	2	2	2	2	2	2
Inhibition of biofilm formation for *S. mutans*	2	2	2	2	2	0	1	0	2	2	2	0
Inhibition of biofilm formation for *C. albicans*	2	2	2	1	2	1	1	1	2	1	2	1
Obtained score	**24**	**24**	**21**	**15**	**26**	**17**	**21**	**19**	**25**	**24**	**24**	**19**
Maximum score	35											

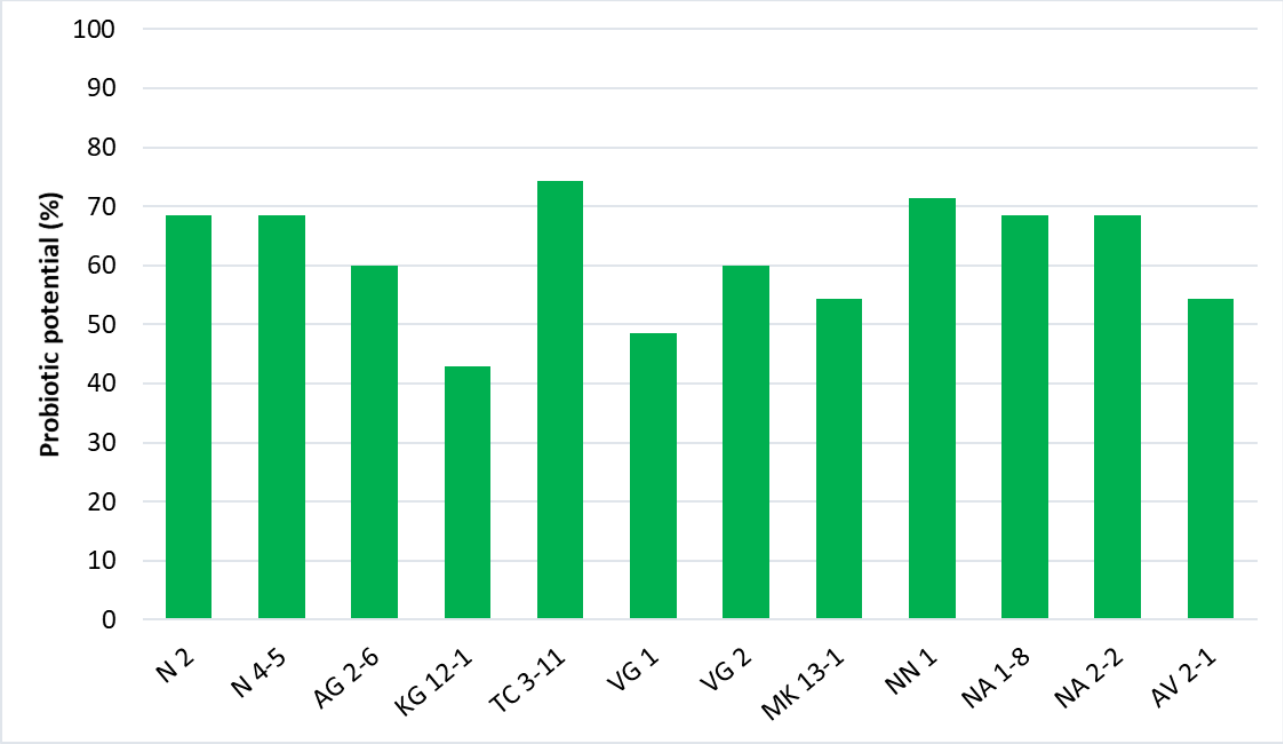

Figure 15 – Probiotic potential of the tested LAB strains. Strains: *L. fermentum* N 2, *L. fermentum* N 4-5, *W. confusa* AG 2-6, *L. curvatus* KG 12-1, *L. fermentum* TC 3-11, *L. delbrueckii* subsp. *sunkii* VG 1, *L. delbrueckii* subsp. *lactis* VG 2, *L. delbrueckii* subsp. *lactis* MK 13-1, *W. confusa* NN 1, *L. rhamnosus* NA 1-8, *L. fermentum* NA 2-2, and *L. paracasei* AV 2-1.

9.2. Application of the selected strains in different model products as fresh concentrated culture and viability evaluation

Preservation of the viability of LAB is an important factor to effectively achieve the desired effects when they are consumed as part of the formulation of probiotic products. Maintaining high cell density is ensured by different food matrices, with a specific component composition and in a specific storage and administration form – sachets, capsules, tablets, candy, etc. Depending on this, varying degrees of protection is provided against factors that are responsible for loss of cell viability during storage, including temperature, high water content and oxygen (Wilcox et al., 2020).

The viability of the two selected strains was evaluated after incorporation as fresh concentrated culture into two types of model product and after storage for 90 days at 4°C. The results for the cocoa-based model product showed that immediately after moulding, the initial cell concentration of *L. fermentum* N 2 was measured at 10^9 CFU/g, while the initial concentration of *L. delbrueckii* subsp. *lactis* VG 2 was measured at 10^8 CFU/g (Figure 16). During storage, *L. fermentum* N 2 maintained cell density up to day 30 with a log value of 10^8 CFU/g, and up to day 90, cell density was maintained with a log value of 10^7 CFU/g. For *L. delbrueckii* subsp. *lactis* VG 2, viability was maintained with a log value of 10^6 CFU/g from day 10 to day 90 of storage.

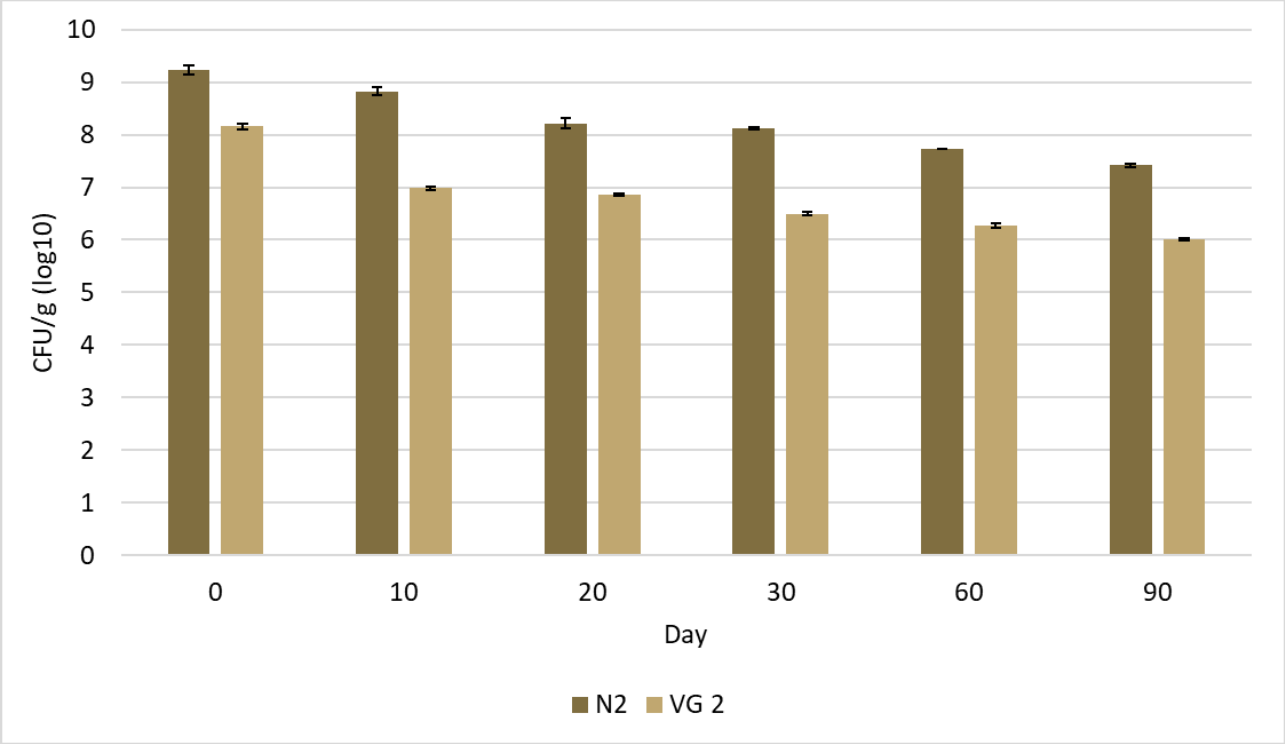

Figure 16 – Viability of the selected LAB strains when incorporated as a fresh culture into a cocoa-based model product and stored at 4°C for 90 days. Values are expressed as mean ± SD. One-way ANOVA + post-hoc Tukey test ($p < 0.01$). Strains: *L. fermentum* N 2, *L. delbrueckii* subsp. *lactis* VG 2.

The results for the agar-based model product showed that immediately after moulding, the initial cell concentration of *L. fermentum* N 2 was measured at 10^8 CFU/g and the initial cell concentration of *L. delbrueckii* subsp. *lactis* VG 2 was measured at 10^6 CFU/g (Figure 17). During storage, the samples with *L. fermentum* N 2 maintained cell density up to day 30 with a log value of 10^7 CFU/g and for the later time samples at day 60 the cell density was measured at 10^6 CFU/g and at day 90, a concentration of 10^5 CFU/g was reported. For *L. delbrueckii* subsp. *lactis* VG 2, viability was maintained at 10^6 CFU/g only until day 10 of storage. Cell density of 10^4 CFU/g was measured after day 20, 10^3 CFU/g on day 30, and 10^1 CFU/g on day 60. At day 90, no viability of the tested strain was reported in the agar model product.

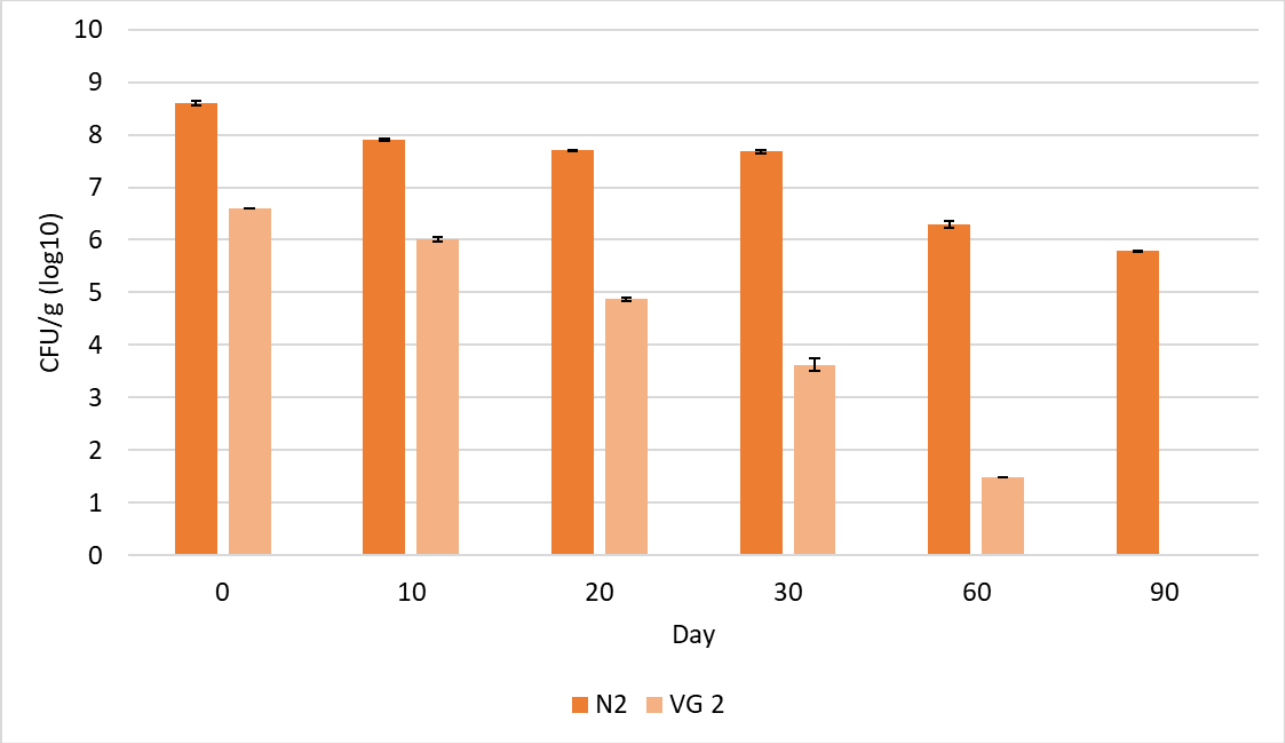

Figure 17 – Viability of the selected LAB strains when incorporated as a fresh culture into an agar-based model product and stored at 4°C for 90 days. Values are expressed as mean ± SD. One-way ANOVA + post-hoc Tukey test ($p < 0.01$). Strains: *L. fermentum* N 2, *L. delbrueckii* subsp. *lactis* VG 2.

From the obtained data, it can be reported that in the cocoa-based model product, both studied strains retained stable viability, with a decrease in their cell density with up to two logs over the entire period of storage. While for the agar model product, *L. fermentum* N 2 showed a decrease in cell density of up to three logs for the entire storage period, and *L. delbrueckii* subsp. *lactis* VG 2 showed a distinct decrease in CFU. This determines that the model cocoa product is a more suitable matrix for maintaining the stability and viability of the probiotic strains included as fresh cultures in its composition.

In a study by Foong et al., the authors evaluated an *L. plantarum* strain included in a high cocoa content model product. A difference of less than two logs from the initial concentration in the product was reported when in storage for three months (Foong et al., 2013). Succi et al. incorporated strains of *L. casei* DG, *L. paracasei* F19, *L. rhamnosus* GG, and *L. reuteri* DSM17938 to a cocoa product and reported the survival of the individual strains after 90 days of storage. The results showed viability of *L. paracasei* F19 and *L. rhamnosus* GG with a log value of 10^8 CFU/dose, *L. casei* DG with a log value of 10^7 CFU/dose, and *L. reuteri* DSM17938 with a log value of 10^6 CFU/dose until the end of the storage period (Succi et al., 2017). A study by Hossain et al. evaluated the survival of *L. delbrueckii* subsp. *bulgaricus* strains icorporated into a cocoa product. From the results, the authors reported that the survival of the strains was higher when stored at 4°C and within 90 days, a difference of 3–5 logs was reported from the starting concentration in the product (Hossain et al., 2022).

9.3. Application of the selected strains in a model product in freeze dried state and viability evaluation

The potential for maintaining the viability of the two selected strains was investigated when incorporated in lyophilized form into an agar formulation of a model product and after storage for 90 days at 4°C. Components were initially selected and a suitable lyoprotective medium was constructed in order to obtain a freeze-dried form from the two studied strains and which would also be suitable for incorporation into the final model product. In the first stage of this experimental task, the survival of the two strains in a freeze-drying process with the applied new protective medium was evaluated. The results showed that the constructed lyoprotectant maintained the viability of the strains after freeze-drying (Figure 18).

Figure 18 – Viability of the selected LAB strains in a freeze-drying process with the newly-constituted protective medium. Values are expressed as mean ± SD. One-way ANOVA + post-hoc Tukey test: a – $p > 0.05$; c – $p < 0.01$, respectively. Strains: *L. fermentum* N 2, *L. delbrueckii* subsp. *lactis* VG 2.

Preserved cell density was reported by a log value of 10^9 CFU/mL for *L. fermentum* N 2, while cell concentration was reduced by less than one log for *L. delbrueckii* subsp. *lactis* VG 2. The results demonstrate that the newly designed lyoprotective medium provides high stability of the LAB strains during the freeze-drying process.

Freeze-dried cultures of the two selected strains were incorporated into an agar model product. Immediately after incorporation, an initial cell concentration of 10^7 CFU/g was reported for *L. fermentum* N 2 and an initial concentration of 10^8 CFU/g was reported for *L. delbrueckii* subsp. *lactis* VG 2 (Figure 19). During storage, at day 10, *L. fermentum* N 2 maintained its cell density unchanged, and at later time points, at day 30 it was 10^6 CFU/g,

at day 60 and 90 cell density was measured at 10^5 and 10^4 CFU/g, respectively. For *L. delbrueckii* subsp. *lactis* VG 2, a decrease in viability of about one log was observed at each time point of measurement, with concentration of 10^3 CFU/g recorded at day 90 for this strain.

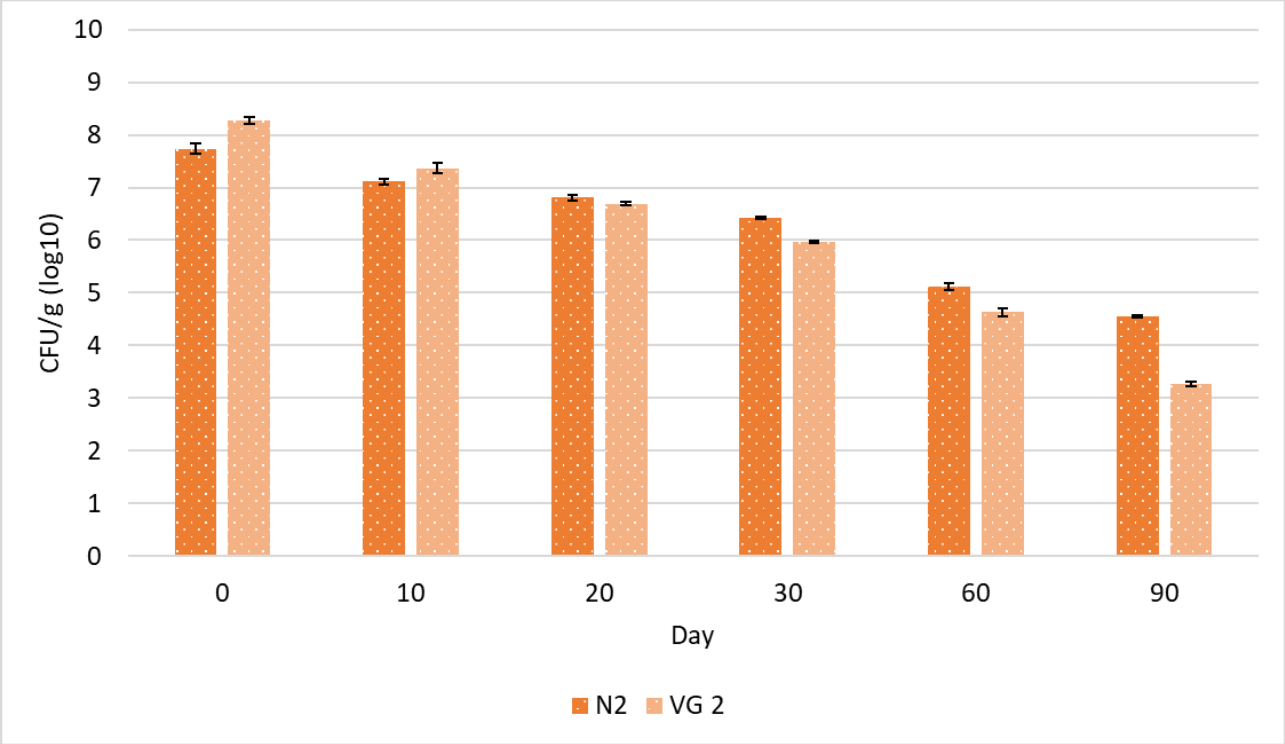

Figure 19 – Viability of the selected LAB strains when incorporated in a freeze-dried form into an agar-based model product and stored at 4°C for 90 days. Values are expressed as mean ± SD. One-way ANOVA + post-hoc Tukey test ($p < 0.01$). Strains: *L. fermentum* N 2, *L. delbrueckii* subsp. *lactis* VG 2.

From the obtained data, it was found that the preliminary freeze-drying improved the stability and survival of both strains when introduced into the model agar product. *L. fermentum* N 2 was observed to retain its stability in both fresh and freeze-dried forms, with both agar model products showing up to three logs difference at day 90 from the initial culture concentration. For *L. delbrueckii* subsp. *lactis* VG 2, it is distinctly noticeable that the incorporation of the strain into the agar product in freeze-dried form improved the stability and survival of the strain. The initial cell concentration of the culture when incorporated into the product was reported to be two logs higher compared to the initial concentration when added in fresh form. Also, higher viability values were reported throughout the storage period with survival also observed at day 90.

In a study by Zavistanaviciute et al., the authors evaluated several LAB strains added into chewy candy formulations in a freeze-dried form. From the results, they found that after storage for nearly one month, a difference of 1–2 logs in the viability of the strains was reported, relative to the starting concentration in the product (Zavistanaviciute et al., 2022).

9.4. Side microflora evaluation of the prepared model products

Probiotic products need to go through various stages of quality control to ensure their safe use. Microbiological control tests include not only the determination of the total viable cell count of probiotic microorganisms, but also microbiological purity tests (Zavišić et al., 2023). Probiotic products, depending on the category of the specific product group, are monitored for microbiological purity and safety by tracking the absence of indicator contaminants such as *E. coli* and *Salmonella* sp., and the requirements for each individual product group are regulated by the European and American Pharmacopoeia, the Food and Drug Administration and the European Commission (Zawistowska-Rojek et al., 2022). Depending on the route of administration, maximum total microbial counts for aerobic microorganisms, and yeasts and moulds are established for products containing live microorganisms. For control purposes, a variety of culture media and incubation conditions are used that are specific to the product under investigation and the microorganisms therein (Zawistowska-Rojek et al., 2022).

The prepared model products were analyzed for total saprophytic microorganisms and *E. coli* (Table 15). For the indicator total number of saprophytic microorganisms, the two agar-based model products did not show the presence of side microflora in the samples prepared for analysis until the 90th day of storage. For the cocoa-based model product, which was prepared with starting materials that had not undergone a preliminary sterilisation step, the presence of side microflora was reported in the samples with *L. fermentum* N 2 up to day 60 and in the samples with *L. belbrueckii* subsp. *lactis* VG 2 up to day 90, with a total aerobic microbial count of less than 100 CFU/g (Table 15). None of the samples of model products prepared for analysis showed the presence of *E. coli* and, according to the accepted standards of practice, these results are expressed as < 10 CFU/g.

Table 12 – Evaluation for total saprophytic microorganisms and *E. coli* in the finished model products after storage at 4°C for 90 days.

Product	CFU/g	day					
		0	10	20	30	60	90
Cocoa product + N 2 (fresh culture)	Saprophytic MO	< 100	< 100	< 100	< 100	< 100	< 10
	E. coli	< 10	< 10	< 10	< 10	< 10	< 10
Agar product + N 2 (fresh culture)	Saprophytic MO	< 10	< 10	< 10	< 10	< 10	< 10
	E. coli	< 10	< 10	< 10	< 10	< 10	< 10
Agar product + N 2 (freeze-dried form)	Saprophytic MO	< 10	< 10	< 10	< 10	< 10	< 10
	E. coli	< 10	< 10	< 10	< 10	< 10	< 10
Cocoa product + VG 2 (fresh culture)	Saprophytic MO	< 100	< 100	< 100	< 100	< 100	< 100
	E. coli	< 10	< 10	< 10	< 10	< 10	< 10
Agar product + VG 2 (fresh culture)	Saprophytic MO	< 10	< 10	< 10	< 10	< 10	< 10
	E. coli	< 10	< 10	< 10	< 10	< 10	< 10
Agar product + VG 2 (freeze-dried form)	Saprophytic MO	< 10	< 10	< 10	< 10	< 10	< 10
	E. coli	< 10	< 10	< 10	< 10	< 10	< 10

For the model cocoa-based product, the reported side microflora was introduced with the raw cocoa mass, as it is not subjected to high-temperature processing due to its nature, but according to the accepted national and European requirements for products containing live microorganisms that have not undergone heat treatment, the permissible titre of total saprophytic microflora should not exceed 1000 CFU/g (Enikova, NCPHA, in Bulgarian). According to the results, all of the three variants of model products incorporated with the two selected strains can be defined as safe for use.

❖❖❖

In conclusion, it can be summarized that the investigated newly isolated LAB strains exhibit probiotic potential and are suitable for inclusion in model probiotic products. The selected strains *L. fermentum* N 2 and *L. delbrueckii* subsp. *lactis* VG 2 were reported to possess survival rates when incorporated into the formulated model products, both as fresh cultures in a cocoa-based product and in freeze-dried form in an agar-based product. The obtained results are a significant prerequisite for continuing and expanding the research with the newly isolated strains and the experimental work to develop new products to improve oral health.

V. Conclusions

1. Taxonomic characterisation of the twelve newly isolated strains showed that four strains belong to the species *Limosilactobacillus fermentum*, one strain to *Latilactobacillus curvatus*, one strain to *Lactobacillus delbrueckii* subsp. *sunkii*, two strains to *Lactobacillus delbrueckii* subsp. *lactis*, one strain to *Lacticaseibacillus rhamnosus*, one strain to *Lacticaseibacillus paracasei* and two strains to the species *Weisella confusa*.
2. All tested strains exhibit peptidase activities and possess specific set of peptidase genes, which determines their potential for production of biologically active peptides.
3. All of the studied strains show a high level of survival under *in vitro* conditions simulating the oral cavity, and most of them grow successfully in the presence of lysozyme, which determines the potential for association in the oral microbiome and the possibility of manifesting their probiotic properties.
4. Stress factors in the next departments of the GIT affect the growth of the studied strains, but most of them successfully survive under the direct influence of these factors.
5. All of the studied strains exhibited strong adhesion potential, assessed as the ability to bind to mucin and the presence of genetic determinants for adhesive proteins, as well as strain-specific characteristics for auto-aggregation and biofilm formation.
6. The newly isolated strains exhibit a broad spectrum of antibiotic multiresistance with a coefficient between 0.385 and 0.615, but the bioinformatics analyses of the whole genome sequences show that they do not possess acquired antibiotic resistance genes.
7. Total antioxidant capacity was established for all studied strains, and expressed antioxidant activity was reported for the cell-free supernatants obtained from seven of the strains.
8. Strain-specific antimicrobial activity was determined for the studied strains, and genetic determinants for bacteriocin production were detected for six of the strains.
9. Eleven of the studied strains showed expressed antagonistic activity against *S. mutans*, and inhibitory activity against *C. albicans* was reported for eight of the strains during co-culturing with these oral test pathogens.
10. The studied strains exhibit coaggregation ability with the oral test pathogens *S. mutans* and *C. albicans* and species-specific antibiofilm activity against these pathogens.
11. The twelve strains showed very good survival after the freeze-drying with two types of constructed protective media, as most of the strains showed better results in the protective media with added trehalose.
12. Using a complex analytical approach, probiotic potential above 60 % was determined for eight of the studied strains.
13. The selected strains *L. fermentum* N 2 and *L. delbrueckii* subsp. *lactis* VG 2 maintained a very good survival rate when incorporated into model products as fresh cultures in a cocoa-based product and in freeze-dried form in an agar-based product, and for a storage period of up to 20 days.

VI. Contributions

1. Twelve new strains were isolated from the human oral microbiome and identified using a complex molecular approach.

2. The genomes of the twelve newly isolated strains were sequenced and the processed whole-genome sequences were deposited in the NCBI genetic database.

3. The direct antagonistic activity of the studied strains against the oral pathogens *Streptococcus mutans* and *Candida albicans* was proven by a combination of analytical methods of co-cultivation, co-aggregation and anti-biofilm activity, and for the first time such results were presented for strains of the species *Latilactobacillus curvatus, Lacticaseibacillus rhamnosus, Lacticaseibacillus paracasei* and *Weissella confusa*.

4. A complex approach was adapted to evaluate the probiotic potential of the newly isolated LAB strains by applying a newly structured scoring system.

5. New model probiotic products have been formulated with the successful inclusion of two selected strains, *L. fermentum* N 2 and *L. delbrueckii* subsp. *lactis* VG 2, which provides a very good basis for the development of products to improve oral health.

VII. Scientific publications

1. Atanasov, N.; Evstatieva, Y.; Nikolova, D. Probiotic Potential of Lactic Acid Bacterial Strains Isolated from Human Oral Microbiome. *Microbiol. Res.* **2023**, *14(1)*, 262–278. doi: 10.3390/microbiolres14010021, IF (1.5 – 2022)

2. Atanasov, N.; Evstatieva, Y.; Nikolova, D. Antagonistic Interactions of Lactic Acid Bacteria from Human Oral Microbiome against *Streptococcus mutans* and *Candida albicans*. *Microorganisms* **2023**, *11(6)*, 1604. doi: 10.3390/microorganisms11061604, IF (4.5 – 2022), Web of Science Qartile: Q2 (2022), SJR (0.909 – 2022), SCOPUS Qartile: Q2 (2022)

3. Atanasov, N.; Trifonova, E.; Evstatieva, Y.; Nikolova, D. Effect of Two Lyoprotectants on the Survival Rate and Storage Stability of Freeze-Dried Probiotic Lactic Acid Bacterial Strains. *JCTM* **2023**, *58(6)*, 1003–1010. doi: 10.59957/jctm.v58i6.1, SJR (0.196 – 2022), SCOPUS Qartile: Q3 (2022)