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FUNCTIONAL AND TECHNOLOGICAL CHARACTERISTICS OF NEWLY ISOLATED LACTIC ACID BACTERIA STRAINS FROM TRADITIONAL FOODS

ABSTRACT

OF PhD THESIS

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

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The dissertation is scheduled for defense by order of the Rector of the University of St. Kliment Ohridski"..... and the defense will be held on ..... at ..... h. in front of a Scientific Jury in composition:

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

LIST OF ABBREVIATIONS

LAB – Lactic acid bacteria
GRAS - Generally Recognized as Safe
QPS – Qualified Presumption of Safety
FDA – The Food and Drug Administration
EFSA – European Food Safety Authority
GIT – Gastrointestinal tract
MRS – De Man–Rogosa–Sharpe media
SM – Skimmed milk media
KDA – Potato dextrose agar media
MEA – Malt extract – agar media
BHI – Brain heart infusion media
PBS – Phosphate-buffered saline
PS – Physiological saline
BSA – Bovine Serum Albumin
DNA – Deoxyribonucleic acid
PCR – Polymerase chain reaction
MAR – Multiantibiotic resistance
NCBI – The National Center for Biotechnology Information
CLSI – Clinical Laboratory Standard Institute
BDS - The National Standard for Bulgarian Yogurt
CFS – Cell-free supernatant
NCFS – Neutralized cell-free supernatant
HHV – Human herpesvirus
MTC – Maximum tolerated concentration
MDBK – Madin–Darby bovine kidney
MOI - Multiplicity of infection
CC – Cytotoxic concentration
IC/EC – inhibition effective concentration
SI – Selective index
OD – Optical density
SD – Standard deviation
RP – Redox potential
TA – Titratable acidity
WHC – Water holding capacity
NMR – Nuclear Magnetic Resonance
OPLS-DA - Orthogonal Discriminant Partial Least Squares Analysis

UNITS:
CFU/ml or CFU/g – Colony forming unit per millilitre (ml) or gram (g)
mV – millivolt
cP – centipoise (1cP = 1 mPa · S)

INTRODUCTION

Traditional foods are of great importance to consumers. These foods are also attracting attention from scientific researchers, as they are a rich source of nutrients and microbiota of interest for scientific research. Studying their characteristics and achieving the production of products with characteristics very similar to theirs is the main challenge nowadays, especially for foods that have health benefits.

Fermented foods are considered foods with health benefits because of the presence of lactic acid bacteria (LAB). LABs are promising sources for the development of new products, especially those that can meet the growing consumer needs for natural products and functional foods. The FDA and EFSA authorities have given them the status referred to as GRAS (Generally Recognized as Safe) and QPS (Qualified Presumption of Safety). *Lactobacilli* are found in a wide range of habitats - inhabiting plants, fruits, dairy and meat environments, juices, fermented beverages, cereals, and grain products. The use of LAB in the preparation of various groups of fermented foods dates back to ancient times. Nowadays, LAB can be used in the dairy industry, as starter cultures in the production of various dairy products, but also participate in the fermentation processes taking place in the silage of fodder, canning of fruits and vegetables, production of bread, meat, alcoholic products etc. They are applied as a starter culture for fermented foods and beverages because, in addition to being able to control the fermentation process, they can improve nutritional, organoleptic, technological characteristics and shelf life. They give additional taste and aroma qualities to fermented products and have preservative properties due to the production of biologically active substances with antimicrobial properties (lactic acid, acetic acid, bacteriocins, etc.).

With the development of technology, a wide variety of strains from the family *Lactobacillaceae, Bifidobacteriaceae*, and *Streptococcaceae* are widely used, both in the production of fermented foods and as probiotics with established characteristics and proven safety. For newly isolated *lactobacilli* strains to be included in starter cultures and/or probiotic products and to become commercially available, they need to be studied concerning a spectrum of functional characteristics, health benefits, safety, and technological characteristics. The selection of strains must go through the step of correct identification to continue with the subsequent steps of characterization, including enzymatic activity, which is related to both the characteristics of the product, such as aroma, taste, texture and the benefits of the host. *Lactobacilli* must adapt to the environment and be able to survive for a long time in the GIT, which is affected by the various conditions (pH of the stomach, digestive enzymes and bile salts, the conditions in the lower parts of the GIT, the abilities of the LAB itself for resistance to impacts, for adhesion, etc.). About 70% of the immune system is located in the gut-associated lymphoid tissue of the GIT. Specific strains of probiotics can influence a wide range of immune functions in health and disease and can be effectively used to optimize human health.

AIM AND TASKS

Over the past three decades, huge research progress has been made in the study of lactic acid bacteria and their spectrum of functional and probiotic characteristics. A wide variety of products containing probiotic bacteria from this group have been developed and put into practice. Despite these advances, interest in lactic acid bacteria research continues to grow, by isolating and characterizing new strains from diverse sources and developing new functional products with health benefits for consumers.

The aim of this dissertation is related to:

Isolation of new strains of lactic acid bacteria from the microbiota of traditionally prepared dairy, meat, and spontaneously fermented products and the study of their functional and technological characteristics for application in new food products with improved functional characteristics and health effects.

To achieve the set goal, the following tasks have been formulated:

1. Isolation of new LAB strains from the microbiota of traditional fermented food products:

- *Collection of samples of fermented products prepared by traditional technology and isolation of new strains of LAB;*
- *Determination of phenotypic and biochemical characteristics of the obtained isolates;*
- *Identification of the newly isolated strains.*

2. Study of functional and probiotic properties of the newly isolated LAB strains:

- *Study of antimicrobial activity against test-pathogenic bacteria and yeasts, food-associated mold and yeast contaminants;*
- *Screening for antiviral activity of the newly isolated strains;*
- *Determination of the enzyme profile of the studied strains;*
- *Evaluation of antibiotic resistance to the studied strains;*
- *Investigation of autoaggregation, coaggregation potential, hydrophobicity, and adhesive abilities of the newly isolated strains;*
- *Determination of the ability of newly isolated strains to survive in conditions simulating different departments of the GIT.*

3. Determination of the main technological characteristics of the studied strains:

- *Determination of total acid-forming capacity;*
- *Assessment of stability in the technological process of Freeze-drying and selection of appropriate protective media.*

4. Inclusion of selected strains from the new isolates in a model product and determination of the main characteristics of the product:

- *Selection of strains and obtaining a model product;*

- *Study of basic physicochemical characteristics and metabolic profile of the obtained lactic acid products during the fermentation process and the storage period;*
- *Evaluation of the viability of the strains in the product for a period of storage and of their bioprotective potential by co-cultivation with food-associated pathogens;*
- *Evaluation and analysis of sensory characteristics of model products with selected strains.*

MATERIALS AND METHODS

1. Test-microorganisms

Escherichia coli ATCC 25922, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus cereus* ATCC 11778, *Candida albicans* ATCC 18204, *Aspergillus niger* A3, *Aspergillus flavus* NBIMCC 916, *Fusarium proliferatum* BT 140, *Penicillium claviforme* BT 136, *Kluyveromyces lactis* 1470, *Kluyveromyces marxianus* var t3 and *Saccharomyces cerevisiae* NBIMCC 537.

2. Nutrient media and solutions

Media: MRS broth, MRS agar (De Man, Rogosa & Sharpe medium) (Merck; Oxoid); 10% Sterile Milk (SM) (HiMedia); Potato dextrose agar (KDA) (Oxoid); Malt extract - broth, Malt extract - agar (MEA) (Oxoid); Mueller-Hinton agar (Sigma-Aldrich); BHI agar (Sigma-Aldrich); HiCrome *E. coli* agar (HiMedia). Ready for use dry/granulated nutrient media were prepared according to the manufacturer's instructions; *Solutions:* Physiological saline (PS); PBS-phosphate buffered saline.

3. Isolation, cultivation, and storage of microorganisms

Isolation of pure cultures of lactic acid bacteria; Cultivation of the obtained isolates - on liquid nutrient media MRS-broth and SM or solid medium MRS-agar at a suitable temperature for each isolate. *Storage of the obtained isolates* - in a frozen state at -80 °C and in a freeze-dried form.

4. Physiological, biochemical, and molecular methods

Gram staining; Determination of catalase activity; Determination of oxidase activity; Determination of peroxidase activity; Determination of coagulating ability; Determination of biochemical profile - with the API 50CHL system (bioMérieux);

Isolation of genomic DNA - with the Zymo Research Quick-DNA™ Miniprep Plus Kit; *Sequencing analysis of the 16S rDNA gene* - amplification with universal primers 27F and 1492R, sequencing of the PCR products by Macrogen Inc., The Netherlands. Generated sequences were analysed by BLASTN in the NCBI database;

Determination of the enzyme profile of the studied strains - with the test kit API ZYM Kit (bioMérieux); *Screening for genetic determinants for peptidases* - PCR analysis with primers for peptidases genes pepO, pepN, pepQ, pepR, pepT, pepX and visualization by 1% agarose gel electrophoresis.

5. Determination of the antibiotic resistance profile of the studied strains

Antibiotic susceptibility test - Kirby–Bauer disc diffusion method, data interpreted according to CLSI 2020; *Screening for antibiotic resistance genes* - PCR assay with 28 pairs of specific primers for 12 different antibiotics and visualized by 1% agarose gel electrophoresis.

6. Determination of autoaggregation and coaggregation properties and hydrophobicity - optical density (OD) measurement.

7. Determination of ability to adhere to mucin under *in vitro* conditions - determination of CFU/ml adhered cells.

8. Determining *in vitro* the ability of the studied strains to survive in conditions simulating different departments of the GIT

In vitro assessment of the ability of the studied strains to grow under simulated conditions of different departments of the GIT- at pH2 and pepsin, pancreatic enzymes, and different concentrations of bile salts. An inhibition coefficient (C_{inh}) was calculated, with $C_{inh} \leq 0.40$ considered a significant criterion for determining the tested strains as potential probiotic candidates; *Determination of survival of isolated strains at pH 2 and pepsin and at 0.3% bile salts* – CFU/ml, the percentage of bacterial survival was calculated.

9. Determination of antimicrobial activity

Antibacterial activity - five test pathogens *E. coli, B. subtilis, S. aureus, P. aeruginosa, B. cereus* were used; *Antimicrobial activity against the yeast test pathogen C. albicans; Antagonistic activity against food-associated mold contaminants* - method of direct antagonism against *A. niger, A. flavus, F. proliferatum,* and *P. claviforme; Antagonistic activity against food-associated yeast contaminants* - *K. lactis, K. marxianus* and *S. cerevisiae*, percentage of growth inhibition was determined.

10. Test for antiviral activity

Preparation of cell-free supernatant (CFS); Cytotoxicity test - colorimetric MTT assay. The CC_{50} was calculated by regression analysis of the constructed dose-response curves; *Antiviral activity* - against human herpes virus models HHV-1 and HHV-2 by MTT-based colorimetric assay to detect inhibition of HHV replication. The EC_{50} was calculated by regression analysis of the dose-response curves generated from the data. The Selective Index (SI) is calculated as: CC_{50}/EC_{50}; *Virucidal activity* - by direct contact assay.

11. Methods for determining the main technological characteristics of the studied strains

Determination of total titratable acidity; Freeze-drying processes – in two types of protective media, CFU/ml was determined before, after freeze-drying, and after storing samples at 4°C for 3 and 6 months.

12. Obtaining model yogurt products with selected strains

Model yogurt products – one control variant and three experimental variants. Samples were stored at 4°C and analysed on days 0, 7, 14, 21 and 28.

Table IV.2. Variants of prepared yogurt

Variants of yogurt	Concentration, %		
	Starter culture (LB Bulgaricum))	*L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3	*L. plantarum* KC 5-12
1	1	-	-
2	0.1	5	-
3	0.1	-	5
4	0.1	2.5	2.5

13. Determination of basic physicochemical and organoleptic characteristics of the prepared yogurt model products

Determination of pH, redox potential, and titratable acidity; Determination of water holding capacity (WHC) and syneresis; Determination of viscosity; Sensory analysis - 15

volunteers, seven sensory indicators, which were compared to BDS 12:2010. The conditions for conducting the sensory analysis are in accordance with BDS 15612:1983;

Determination of the total number of LAB in a product during the storage period - CFU/g; *Determination of survival of test pathogen E. coli in co-cultivation with test strains in a product model* - CFU/ml with HiCrome *E. coli* Agar (HiMedia) selective medium for *E. Coli*, before, after fermentation and on day 5 of storage.

14. NMR spectroscopic analyses of the obtained yogurt model products

The analyses were carried out in the Center for NMR Spectroscopy at the IOCCP-BAS on a 600.18 MHz Bruker Avance NEO spectrometer. Quantitative NMR data were statistically processed by orthogonal discriminant partial least squares analysis (OPLS-DA) to distinguish between the four types of yogurt samples.

All assays were performed in triplicate and results are presented as means ± SD. One-way ANOVA analysis with post hoc Tukey test was applied to compare mean values of yogurt samples and storage period.

RESULTS AND DISCUSSION

1. Isolation of new LAB strains from the microbiota of fermented products

1.1. Collection of samples of fermented products prepared by traditional technology and isolation of new strains of LAB

Functional fermented foods, in addition to providing valuable nutritional components, also exhibit beneficial properties for consumers (del Castillo et al., 2018). Since fermented foods are produced in the presence of microorganisms such as LAB, the focus for research on these bacteria has increased. The basis of the studies includes studying the diversity of microbiota in food products, isolating new strains, and determining their characteristics and beneficial properties. The researches have shown that LAB not only exhibits beneficial properties for consumers but also provides changes in final products leading to an increase in their quality (Colombo et al., 2018; Dapkevicius, 2022).

Fermented foods have been produced and consumed by humans for thousands of years, resulting in a huge variety of such products with distinctive regional and cultural specificities. To a significant extent, the microbiota of traditional fermented foods is determined by the geographical and ecological features of the specific regions and by the preserved traditions in their preparation.

With modern industrialization and the increasing demands in the production of fermented foods, the main products of this type are prepared with well-studied and standardized starter cultures in industrial conditions. Collecting samples prepared entirely by traditional methods, in home conditions and without the use of industrial starter cultures, is increasingly difficult. It is very rare to find producers who can confirm the origin of their homemade sourdough starters. Homemade sourdoughs of proven origin and duration of application are a valuable source of strains of LAB that are characteristic of the local ecosystem and have interesting and useful functional characteristics in fermented foods.

For the present research work, different types of traditional fermented foods from the Gora region, of Albania were selected. The Gora region of Albania is defined as mountainous, with extensive agriculture and well-preserved traditions for preparing a variety of fermented foods. As described in the Literature review section, the rich variety of traditional fermented foods for this region is an excellent basis for the selection of diverse products from the microbiota of which to isolate new strains of lactic acid bacteria.

Samples of the products that were selected in the present research work include traditionally prepared: cow's yogurt, goat's yogurt, cow's cheese, goat's cheese, sheep's cheese, local sujuk, and spontaneously fermented fruits. For all selected products, the traditional technology for their preparation was studied and it was found that no commercial starter cultures were used:

- ✓ A sample of cow yogurt - from homemade yogurt according to the traditional method, stored and passed down in the family of a producer from the village of Oreshek, Gora. The sourdough used was kept in home conditions, and the information about its storage and application goes back two generations. According to the producer's family tradition, in the past, sourdough was obtained by using hazelnut twigs with peeled bark, which were introduced into preheated and tempered fresh milk to about 60 ºC. The fermented product thus obtained was used for preparation in a subsequent cycle, and after 3 to 4 consecutive re-fermenting processes, the obtained yogurt was considered fit for consumption.

- ✓ A sample of goat yogurt - prepared at home using a traditional technology, similar to that described in item 1, by a producer from the village of Pakisha, Gora.
- ✓ Sample of goat cheese - goat cheese prepared at home using traditional technology using only rennet yeast (rennet enzyme) from a producer from the village of Pakisha, Gora. In the production of this type of product, the local population had a tradition of using rennet material from the stomachs of young animals, but nowadays rennet yeast is used - a commercial product. The value of these samples lies in the natural microflora that enters the products, both with the starting raw materials and during the ripening processes.
- ✓ A sample of cow's cheese - prepared according to traditional home technology by a producer from the village of Shishteec, Gora.
- ✓ A sample of sheep's yellow cheese - prepared according to traditional home technology by a producer from the village of Shishteec, Gora.
- ✓ A sample of Shishteechki sujuk - a traditional local product that the local population produces from ground beef with additions of vegetable spices and stuffed into previously prepared animal intestines. The product is prepared according to traditional technology by a producer in the village of Shishteec, Gora. This type of product is dried under natural conditions and spontaneous fermentation processes take place inside the meat mass with the participation of the microorganisms that arrived with the raw materials.
- ✓ A sample of a fermented fruit product - fermented pears prepared at home by a producer from the village of Oreshek, Gora. The product is produced traditionally by the local population and is obtained as a result of a spontaneous fermentation process of the fruits in a water environment and without access to oxygen. Under these conditions, a fermentation process takes place with the participation of the microflora, mostly from the fruits themselves, and this product is an interesting source for isolating LAB.

Initially, enriched cultures were obtained from the selected products, from which, after ten-fold dilutions and inoculation, single colonies were obtained. Single colonies with characteristic morphology were selected, from which 12 pure cultures were isolated. They are named: KZM 2-11-1, KZM 2-11-3, KO 3-7-5, KO 4-4, KC 5-12, KC 5-13, KC 5-14, KZC 8- 21-1, KZC 8-23-5, C 10-31-3, KBB 7-1, KBB 11 (Table V.1). All isolates grow well on MRS medium, at the thermophilic or mesophilic temperature presented in Table V.1. No pure cultures were isolated from the cow yogurt sample under the selected experimental conditions.

1.2. Determination of phenotypic and biochemical characteristics of the obtained isolates

To prove the belonging of the new isolates to the LAB group, a series of analyzes were carried out to determine basic morphological and physiological-biochemical characteristics. The initial step for pure cultures is phenotypic characterization. To determine these basic characteristics, fresh microscopic preparations, permanent microscopic preparations with Gram stain, and tests for determination of oxidase, catalase, and peroxidase activity and for coagulation were prepared. Of the newly isolated strains, 11 are characterized by a rod-shaped cell morphology, and one KC 5-13 has a spherical cell shape in pairs. The two strains KZM 2-11-1 and KZM 2-11-3 are characterized as long rods, strains KC 5-12 and KC 5-14 are medium long rods, KO 3-7-5, KO 4-4, KZC 8- 21-1 and KZC 8-23-5 are short rods, and strains C 10-31-1, KBB 7-1 and KBB 11 are characterized as very short rods (Figure V.1).

Table V.1. Pure cultures and their origin from traditionally prepared products

Pure culture	Optimal temperature	Origin
KZM 2-11-1	41°C	Goat's yogurt
KZM 2-11-3	41°C	Goat's yogurt
KO 3-7-5	41°C	Sheep's yellow cheese
KO 4-4	30°C	Sheep's yellow cheese
KC 5-12	41°C	Cow's white cheese
KC 5-13	41°C	Cow's white cheese
KC 5-14	30°C	Cow's white cheese
KZC 8-21-1	37°C	Goat's white cheese
KZC 8-23-5	30°C	Goat's white cheese
C 10-31-3	30°C	Meat (sujuk)
KBB 7-1	30°C	Spontaneously fermented fruits
KBB 11	30°C	Spontaneously fermented fruits

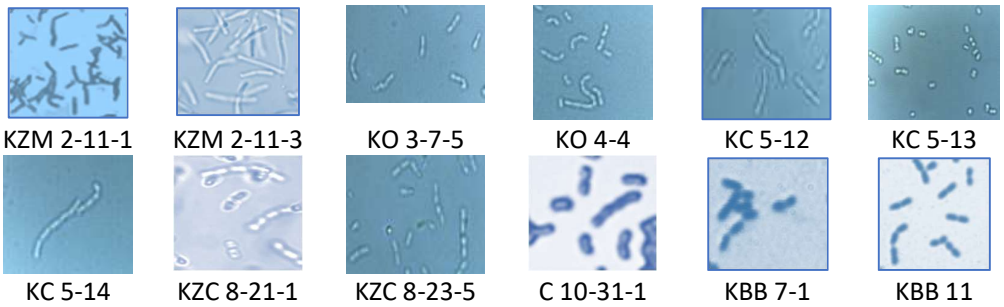


Figure V.1. Morphology of newly isolated LAB strains

The obtained results of Gram staining, screening test for oxidase, catalase, and peroxidase activity, and coagulating ability of the newly isolated strains are presented in Table V.2.

Table V.2. Characteristics of newly isolated strains

Strains	Cell morphology	Gram	Catalase	Oxidase	Peroxidase		Coagulation	
					MRS	SM	16h	24h
KZM 2-11-1	Rod-shaped	+	-	-	-	+	+	+
KZM 2-11-3	Rod-shaped	+	-	-	-	+	+	+
KO 3-7-5	Rod-shaped	+	-	-	-	-	+	+
KO 4-4	Rod-shaped	+	-	-	-	-	+	+
KC 5-12	Rod-shaped	+	-	-	-	-	+	+
KC 5-13	Coccoidal	+	-	-	-	-	+	+
KC 5-14	Rod-shaped	+	-	-	-	-	+	+
KZC 8-21-1	Rod-shaped	+	-	-	-	-	+	+
KZC 8-23-5	Rod-shaped	+	-	-	-	-	+	+
C 10-31-3	Rod-shaped	+	-	-	-	-	-	-
KBB 7-1	Rod-shaped	+	-	-	-	-	+	+
KBB 11	Rod-shaped	+	-	-	-	-	+	+

All strains are gram-positive, catalase, and oxidase-negative, which corresponds to belonging to the group of lactic acid bacteria. Peroxidase activity was found in two strains KZM 2-11-1 and KZM 2-11-3, only when cultured in medium SM. The coagulating ability of the newly isolated strains was determined in SM medium, with coagulation observed up to the 16th hour of incubation in 11 of the newly isolated strains, but no coagulation was observed in strain C 10-31-1 even after 24 hours.

1.3. Identification of isolated strains

Accurate and reliable identification of newly isolated strains is a fundamental and very important stage of the process of their research, especially when their functional characteristics

and potential for applications in different products will be determined. To identify the newly isolated LAB strains to species, a polyphasic taxonomic approach was used, by determining a biochemical profile with the API® 50 CHL test kits (bioMérieux, France) and sequencing analysis of the 16S rDNA gene (Table V.3). The obtained results of determining the biochemical profile show that out of a total of 12 strains in total, 9 of them are identified with a credibility level of over 98.8% (Appendix 1). Two strains were identified as belonging to the species *Lactobacillus delbrueckii* ssp. *bulgaricus*, showing the characteristic profile of this species. One strain was identified as belonging to the species *Pediococcus pentosaceus* 1, and 6 strains to the species *Lactobacillus plantarum* 1. Strain KO 4-4 was identified as *Lactobacillus plantarum* 1, but with a low credibility of 58.9%, and due to the low credibility rate, this strain was assumed not to be identified. Two of the strains KO 3-7-5 and C 10-31-3 were not identified by biochemical profile.

Table V.3. Identification of newly isolated strains

Strains	By biochemical profile, with the API WEB	Sequencing analysis of the 16S rDNA gene
KZM 2-11-1	*Lactobacillus delbrueckii* ssp. *bulgaricus*	*Lactobacillus delbrueckii* ssp. *bulgaricus*
KZM 2-11-3	*Lactobacillus delbrueckii* ssp. *bulgaricus*	*Lactobacillus delbrueckii* ssp. *bulgaricus*
KO 3-7-5	Not identified	*Loigolactibacillus coryniformis*
KO 4-4	Not identified	*Lactiplantibacillus plantarum*
KC 5-12	*Lactiplantibacillus plantarum*	*Lactiplantibacillus plantarum*
KC 5-13	*Pediococcus pentosaceus*	*Pediococcus pentosaceus*
KC 5-14	*Lactiplantibacillus plantarum*	*Lactiplantibacillus plantarum*
KZC 8-21-1	*Lactiplantibacillus plantarum*	*Lactiplantibacillus plantarum*
KZC 8-23-5	*Lactiplantibacillus plantarum*	*Lactiplantibacillus plantarum*
C 10-31-3	Not identified	*Latilactibacillus sakei*
KBB 7-1	*Lactiplantibacillus plantarum*	*Lactiplantibacillus plantarum*
KBB 11	*Lactiplantibacillus plantarum*	*Lactiplantibacillus plantarum*

In 2020, a new classification of the family *Lactobacillaceae* was introduced. In the new classification, the genera *Lactobacillus*, *Paralactobacillus*, and *Pediococcus* were updated, and 25 new genera were formed based on species included in the previous genus *Lactobacillus* (Zheng, J. et al., 2020). According to the new classification and as a result of the sequence analysis of the 16S rDNA gene (Table V.3), 7 of the newly isolated strains belong to the species *Lactiplantibacillus plantarum* with strain identification KO 4-4, KC 5-12, KC 5-14, KZC 8-21-1, KZC 8-23-5, KBB 7-1 and KBB 11. The presence of *Lactiplantibacillus plantarum* strains in such types of fermented products is an expected result (Behera et al., 2018).

Strains KZM 2-11-1 and KZM 2-11-3 were identified as *Lactobacillus delbrueckii* ssp. *bulgaricus*. These two strains were isolated from traditionally prepared yogurt, which is also an expected result. The presence of such strains in foods contributes to specific properties, such as aroma development, specific taste, and color, including the texture and consistency of the resulting products (Glusać et al., 2015).

The KO 3-7-5 strain was identified as *Loigolactobacillus coryniformis*. This strain was isolated from cheese. Strains of this species are found at low frequency in fermented foods, but there is evidence that they are isolated in goat's milk, for example, the strain with probiotic potential *L. coryniformis* CECT 5711 (Martin et al., 2005).

Strains of the species *Pediococcus pentosaceus* have been isolated from various fermented foods and are often used for the production of meat products and some types of cheese as part of

starter cultures (Gurira et al., 2005; Vidhyasagar et al., 2013; Zommiti et al., 2018; Zhang et al., 2020). From the newly isolated strains, KC 5-13 was identified as *Pediococcus pentosaceus*.

One strain C 10-31-3, identified as *Latilactobacillus sakei*, was isolated from the traditional meat product (sujuk). Strains of this species have been isolated by other researchers from fermented meat, fish products, and rice wine (Tsuji et al., 2018; Najjari et al., 2008) and used as starter cultures for raw dried and smoked products (Najjari et al., 2020; Kobylyatsky et al., 2021).

2. Study of functional and probiotic properties of the newly isolated LAB strains

2.1. Antimicrobial activity against test pathogenic bacteria and yeasts

A very well-known and important role of different LAB strains in their use is to protect against various microorganisms, including food spoilage. This ancient yet modern approach allows food to be preserved naturally and inhibits the development of a spectrum of food-associated pathogens. The use of LAB as biopreservatives ensures the shelf life of foods and guarantees their safety and quality. LAB can produce antimicrobial compounds such as organic acids (lactic acid, acetic acid), bacteriocins, and other metabolites, preventing food spoilage and pathogen development (Tsuji et al., 2018; Najjari et al., 2008; Najjari et al., 2020; Kobylyatsky et al., 2021; Bartkiene et al., 2020).

The antimicrobial activity of the newly isolated strains was tested using cell-free supernatants from 24-hour cultures against gram-positive and gram-negative bacterial test pathogens and pathogenic yeasts by the agar diffusion method (Table V.4). In the studied strains, the inhibitory effect was established against both gram-positive and gram-negative bacteria. All strains inhibited the growth of *E. coli* and *B. subtilis*, with the most pronounced effect observed in strains *L. plantarum* KBB 7-1 and KBB 11 followed by *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-1 and KZM 2-11-3. In all strains belonging to *L. plantarum* - KO 4-4, KC 5-12, KC 5-14, KZC 8-21-1, KZC 8-23-5, KBB 7-1, KBB 11 and strain *P .pentosaceus* KC 5 -13 an effect against *B. cereus* was observed, and three of the strains from the *species L. plantarum* KO 4-4, KBB 7-1 and KBB 11 reported activity against *P. aeruginosa*. Very similar results are described in the scientific literature, where different LAB strains, including the species we studied, exhibit very well-expressed antibacterial activity against various test-pathogenic bacteria (Gurira et al., 2005; Zommiti et al., 2018; Bartkiene et al. al., 2020; Ricci et al., 2019; Jatmiko et al., 2017; Alebiosu et al., 2017; Unban et al., 2021).

Table V.4. Antimicrobial activity of CFS of LAB against bacterial and yeast test-pathogens

	Test-pathogens					
Strains	***E. coli***	***P. aeruginosa***	***B. subtilis***	***B. cereus***	***S. aureus***	***C. albicans***
KZM 2-11-1	12.5±0.50	-	12.5±0.01	-	-	-
KZM 2-11-3	13.3±0.25	-	13.0±0.00	-	-	-
KO 3-7-5	12.3±0.75	-	11.3±0.03	-	-	-
KO 4-4	11.5±0.25	18.0±0.82	11.0±0.00	16.0±0.82	-	-
KC 5-12	12.3±0.25	-	12.0±0.02	12.3±0.47	-	-
KC 5-13	12.5±0.50	-	11.8±0.25	13.5±0.50	-	-
KC 5-14	11.0±0.01	-	12.0±0.00	15.7±0.47	-	-
KZC 8-21-1	12.3±0.25	-	11.3±0.25	15.0±0.82	-	-
KZC 8-23-5	12.3±0.75	-	12.0±0.00	15.3±1.24	-	-
C 10-31-3	11.5±0.04	-	11.0±0.00	-	-	-
KBB 7-1	17.5±0.25	16.5±0.50	18.5±0.50	12.75±0.25	-	-
KBB 11	17.0±0.01	16.5±0.50	19.0±0.02	14.25±0.25	-	-

No antibacterial activity was found in the tests conducted with the neutralized cell-free supernatant (NCFS) of the 24-hour cultures of the studied strains. Based on these results, it can be

concluded that the newly isolated strains exhibit good bioprotective potential against bacterial pathogens and the activity found in native cell-free supernatants (CFS) is mainly due to the produced low molecular weight organic acids (Unban et al., 2021; Nes et al., 2012). In tests for antifungal activity with CFS against *C. albicans*, no such activity was found.

2.2. *Antagonistic activity against food-associated mold contaminants*

Selected food-associated mold contaminants are associated with food contamination and may cause health problems in consumers. Despite the increased acidity, fermented milk products, as well as other fermented foods, are at risk of contamination with pathogenic, toxigenic, spoilage, or allergenic molds of genera such as *Aspergillus, Fusarium, Penicillium* (Tropcheva et al., 2014).

The results of the tests carried out to evaluate the antifungal activity of the LAB strains are presented in Figure V.2 and Table V.5. The growth of the used test molds of the species *F. proliferatum* and *P. claviforme* was completely inhibited by all the tested strains. Most of the strains of the genus *L. plantarum* have a well-expressed inhibitory effect against the test molds of the genus *Aspergillus*, with *A. niger* being completely suppressed by 4 strains of *L. plantarum* – KO 4-4, KC 5-14, KBB 7-1, and KBB 11, and the remaining strains inhibiting *A. niger* up to 50%. The growth of *A. flavus* was completely inhibited by all strains of the genus *L. plantarum* and by *L. coryniformis* and *P. pentosaceus*. In the strains *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-1 and *L. sakei* C 10-31-3 have shown a lower percentage of inhibition of *A. Flavus*, and *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3 has shown no effect. It is important to note that the strains of the genus *L. delbrueckii* ssp. *bulgaricus* inhibits the development of mold species of the genera *Penicillium* and *Fusarium*.

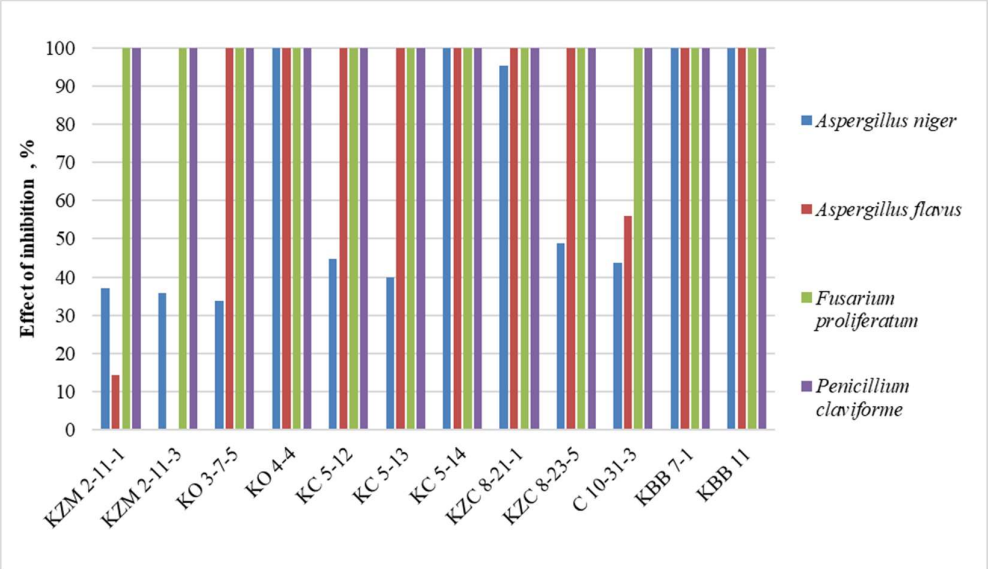
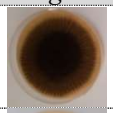
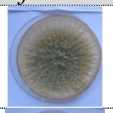
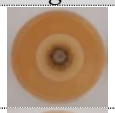
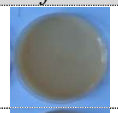
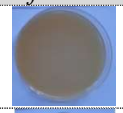
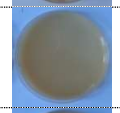
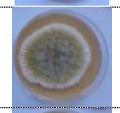
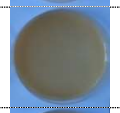
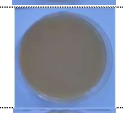
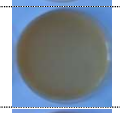
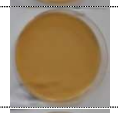
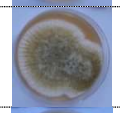
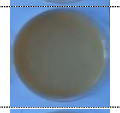
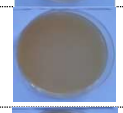
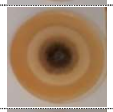
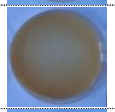
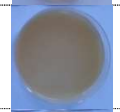
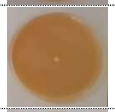
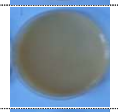
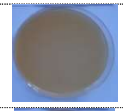
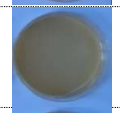
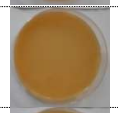
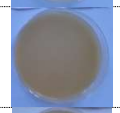
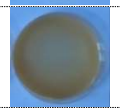
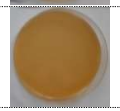
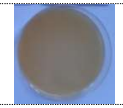


Figure V.2. Antifungal activity of newly isolated strains against food-associated molds expressed as the effect of inhibition, %

The antifungal activity of the studied strains is a promising advantage, suggesting their potential applications as natural food preservatives in various food technologies (Salas et al., 2017). Other studies have shown that different strains of the species *L. plantarum, L. sakei, L. coryniformis* and *P. pentosaceus* exhibit an inhibitory effect against a wide range of pathogens, including *Aspergillus* sp., *Penicillium* sp. and *Fusarium* sp. (Bartkiene et al., 2020; Russo et al., 2017; Syrokou et al., 2021; Tropcheva et al., 2014).

Table V.5. Antifungal activity of the newly isolated LAB strains against food-associated molds represented by inhibition of colony growth

	Test molds					**Test molds**			
LAB strains	*Aspergillus niger*	*Fusarium proliferatum*	*Penicillium claviforme*	*Aspergillus flavus*	LAB strains	*Aspergillus niger*	*Fusarium proliferatum*	*Penicillium claviforme*	*Aspergillus flavus*
Control					KC 5-12				
KZM 2-11-1					KC 5-13				
KZM 2-11-3					KC 5-14				
KO 3-7-5					KZC 8-21-1				
KO 4-4					KZC 8-23-5				
C 10-31-3									
KBB 7-1					KBB 11				

2.3. *Antagonistic activity against food-associated yeast contaminants*

In dairy products, including fresh cheese and yogurt, yeasts of the species *Kluyveromyces marxianus*, *Kluyveromyces lactis,* or *Saccharomyces cerevisiae* are often present (Merchán et al., 2022; Galli et al., 2022; Maïworé et al., 2019). Yeast can metabolize milk components such as lactose, proteins, and fats. They use lactose as a carbon source, competing with LAB for nutrients (Belloch et al., 2011; Kurniawati et al., 2022). They contribute to the characteristics of the product in which they are present due to their ability to produce different aromatic compounds, leading to changes in the final product. LAB are particularly important in fermentation processes because, in addition to producing desirable acids and aromatic compounds, they can inhibit the growth of undesirable organisms (Faria-Oliveira et al., 2015). The presence of yeast imparts different sensory characteristics to the final products and therefore it is necessary to study the activity of LAB to inhibit the growth of unwanted yeast contaminants.

Figures V.3, V.4, and V.5 present the results in % of the growth inhibition effect of the tested yeasts *Kluyveromyces lactis* 1470, *Kluyveromyces marxianus* var t3 and *Saccharomyces cerevisiae* NBIMCC 537 in the presence of CFS from the newly isolated strains. In all three yeast species, growth inhibition was observed depending on the yeast species and CFS from the respective LAB strain. In *Kluyveromyces lactis*, most LAB strains inhibited growth between 22^{-nd} h and 43^{-rd} h, after which this effect decreased as a percentage or completely disappeared (Fig. V.3). Strain *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-1 showed no anti-yeast activity against *Kluyveromyces lactis*.

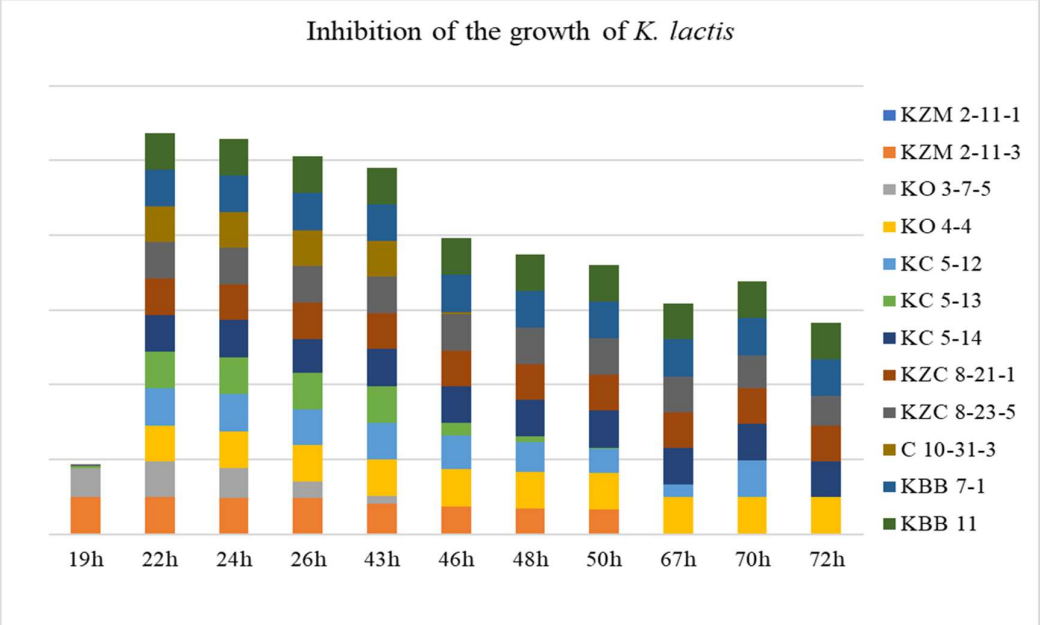


Figure V.3. Inhibition of the growth of *K. lactis* in the presence of CFS of LAB strains

In *Kluyveromyces marxianus*, a more pronounced inhibitory effect was observed after 43 hours (Fig. V.4). Strain *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-1 also showed no activity against *Kluyveromyces marxianus*. In strain *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3, a distinct inhibitory effect appeared at the 26^{-th} hour and increased in the following hours. In the *L. coryniformis* KO 3-7-5 strain, weak activity was observed in the first 26 h, then lost in the following hours. The remaining strains maintained well-expressed activity up to the 72^{-nd} hour of incubation.

The tested LAB strains also inhibited the growth of *S. cerevisiae* for 72 hours, and in some strains, the reported effect decreased or was lost at later hours, and in others the activity was manifested as a well-expressed effect from the 43^{-rd} hour (Fig. V. 5).

Strain *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-1, in which no inhibitory effect was reported in the other two yeast species, showed activity against *S. cerevisiae* for up to 72 hours. Regarding the effect of inhibiting the growth of *S. cerevisiae*, it can be noted that it was better expressed from the 43^{-rd} hour in most of the tested LAB strains.

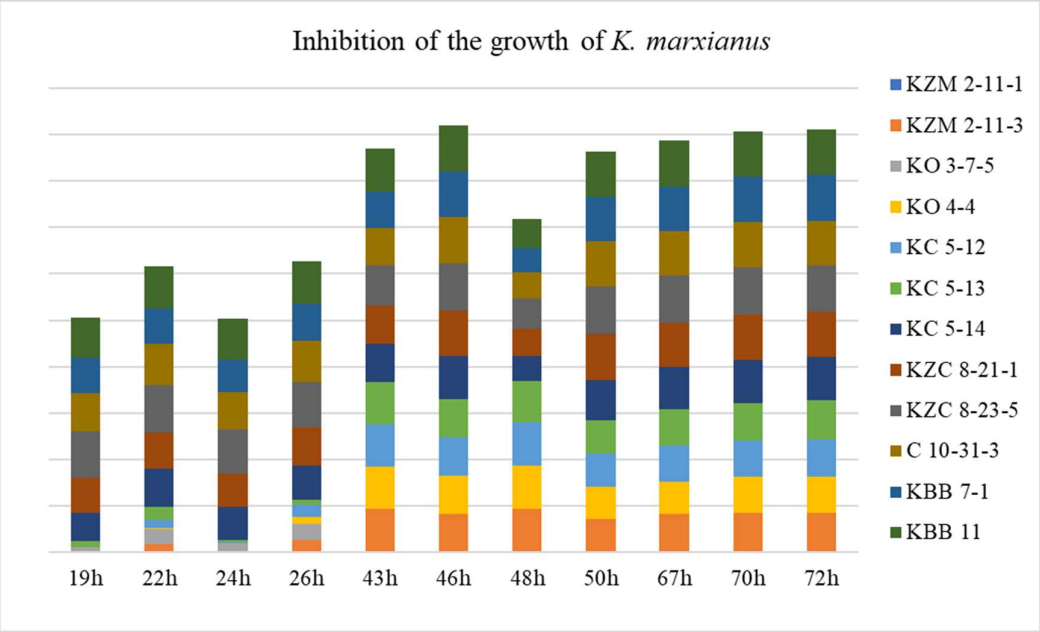


Figure V.4. Inhibition of the growth of *K. marxianus* in the presence of CFS of LAB strains

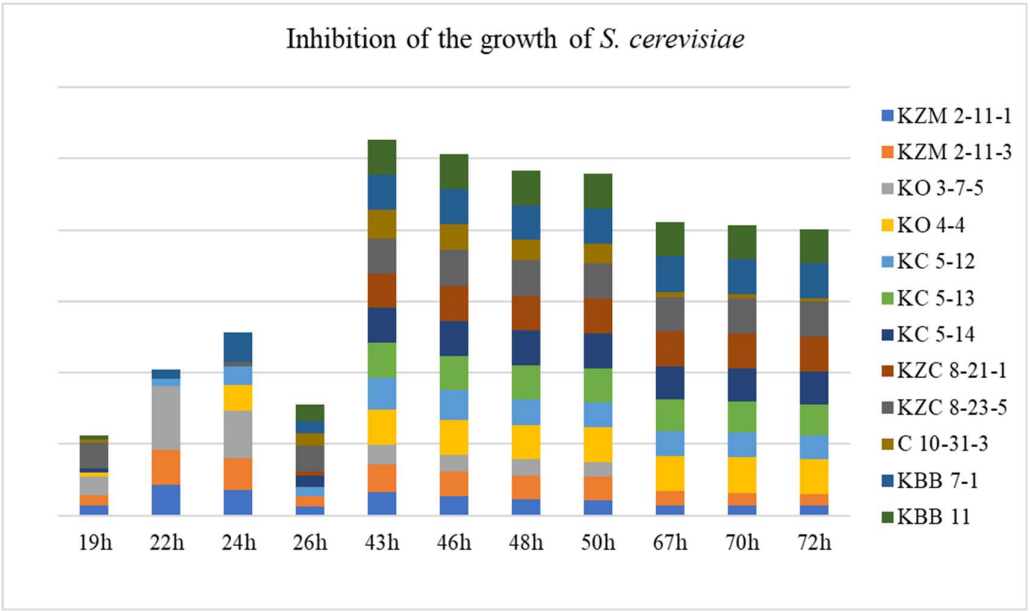


Figure V.5. Inhibition of the growth of *S. cerevisiae* in the presence of CFS of LAB strains

Other studies have presented data for the antagonistic activity of strains of the *L. plantarum* species against *K. marxianus, K. lactis, S. cerevisiae,* and other yeast species (Afzali et al., 2020; Ström et al., 20002). But because common food-associated yeasts have symbiotic relationships

with LAB (Huang et al., 2020; Hu et al., 2023; Chan et al., 2019), researchers have focused more on investigating antimicrobial activity against pathogenic yeasts such as *Candida* sp. (Lipinska-Zubrycka et al., 2020).

2.4. *Screening for antiviral activity of newly isolated LAB strains*

Several authors have reported the antiviral effects of metabolic products of LAB, including from probiotic bacteria, on enveloped and non-enveloped viruses (Zhou et al., 2019; Carver & Naficy 1962; Cliver & Herrmann 1972; Deng & Cliver 1992; Deng & Cliver 1995; Munoz et al., 2011). In different studies, strains isolated from both fermented foods and isolates of human origin have been found to exhibit antiviral activity mainly against influenza viruses and enteroviruses (Muhialdin et al., 2021).

To study the antiviral activity, the concentration of CFS obtained during 24-hour cultivation of the tested LAB, which is not cytotoxic to the MDBK cell culture, should be determined. CFS from twelve samples were applied at concentrations from 100% to 1.5% (Figure V.6) to the MDBK cell line. The maximum tolerated concentration (MTC) was determined microscopically based on morphological changes in the monolayer. All tested CFS had no visible morphological effect on MDBK at a concentration below 25%.

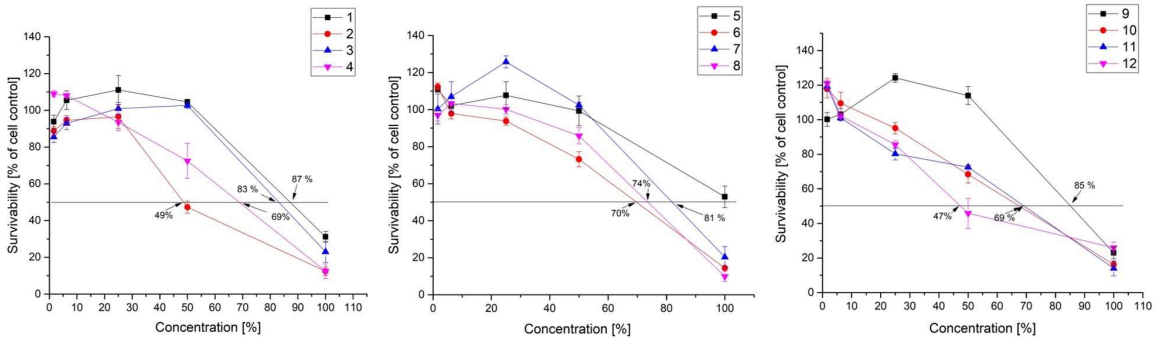


Figure V.6. Cytotoxicity of cell-free supernatants of LAB strains

1. KC 5-12, 2. KC 5-13, 3. KC 5-14, 4. KZC 8-21-1, 5. KZC 8-23 -5, 6. C 10-31-3, 7. KO 4-4, 8. KO 3-7-5, 9. KZM 2-11-3, 10. KZM 2-11-1, 11. KZM 2 -11-3 (SM), 12. KZM 2-11-1 (SM) in MDBK cell line.

The value of the cytotoxic concentration 50 (CC50) was between 47% and 85% in all CFS of the studied LAB strains. The supernatants with the highest toxicity were *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-1 (SM) with a CC50 of 47% and *Pediococcus pentosaceus* KC 5-13 with 49%. Most CFS have a CC50 around 70% - *L. plantarum* KZC 8-21-1, *L. delbrueckii* ssp. *bulgaricus* – KZM 2-11-1 and KZM 2-11-3 (SM medium) (69%), *L. sakei* C 10-31-3 (70%) and *L. coryniformis* KO 3 -7-5 (74%). With lower toxicity are CFS from strains *L. plantarum* KO 4-4 (81%), *L. plantarum* KC 5-14 (83%), *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3 (85%) and *L. plantarum* KC 5-12 (87%). Strain *L. plantarum* KZC 8-23-5 did not achieve 50% of the CC50 (47%) even at the native concentration. The samples obtained after culturing two of the strains on SM medium showed differences in CC50 determination compared to those cultured on MRS medium, for *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-1 it was 47% and for *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3 it was 69 %. These differences may be mainly due to metabolites in the composition of the growth medium on which the studied strains were cultivated. According to Mani-Lopez et al., 2022, one of the main factors that contribute to the component composition of CFS is the initial culture medium. Depending on it, the same strain produces different metabolites. In general, all CFS tested had relatively low cytotoxicity compared to the cell line used in the

assays (Figure V.6). Similar results related to CC50 determination were also confirmed by other studies in which similar cell lines were used – Vero and MDCK (Choi et al., 2009).

Evaluation of inhibition of HHV replication by bacterial cell-free supernatants and determination of the selective index (SI)

To evaluate the inhibition of HHV replication by the tested bacterial CFS, the cell monolayer was treated sequentially with a viral sample and bacterial cell-free supernatant. Cell-free supernatants were used at non-toxic concentrations of 25%, 6.25% and 1.6% and a 50% effective concentration (IC50) was calculated (Figure V.7). The cell-free supernatants in which antiviral activity was reported against both HHV-1 and HHV-2 virus types were from strains *L. plantarum* – KO 4-4, KC 5-12, KC 5-14, KZC 8-23-5, and *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3. Strain *L. plantarum* KC 5-12 had an IC50 of 5% against HHV-1 and less than 1.6% against HHV-2, making it the most effective agent of all twelve samples tested with an SI greater than 54 (Table V.6). In strain *L. plantarum* KC 5-14, a lower efficiency was determined, with an IC50 of 15% and 19%, respectively, but the highest antiviral capacity was reported with nearly 70% inhibition of viral replication and a very clearly expressed dose-dependent effect. For HHV-1, the best efficacy was reported for the cell-free supernatant of *L. plantarum* KO 4-4 with an IC50 of 3.8%, which achieved an SI above 21.

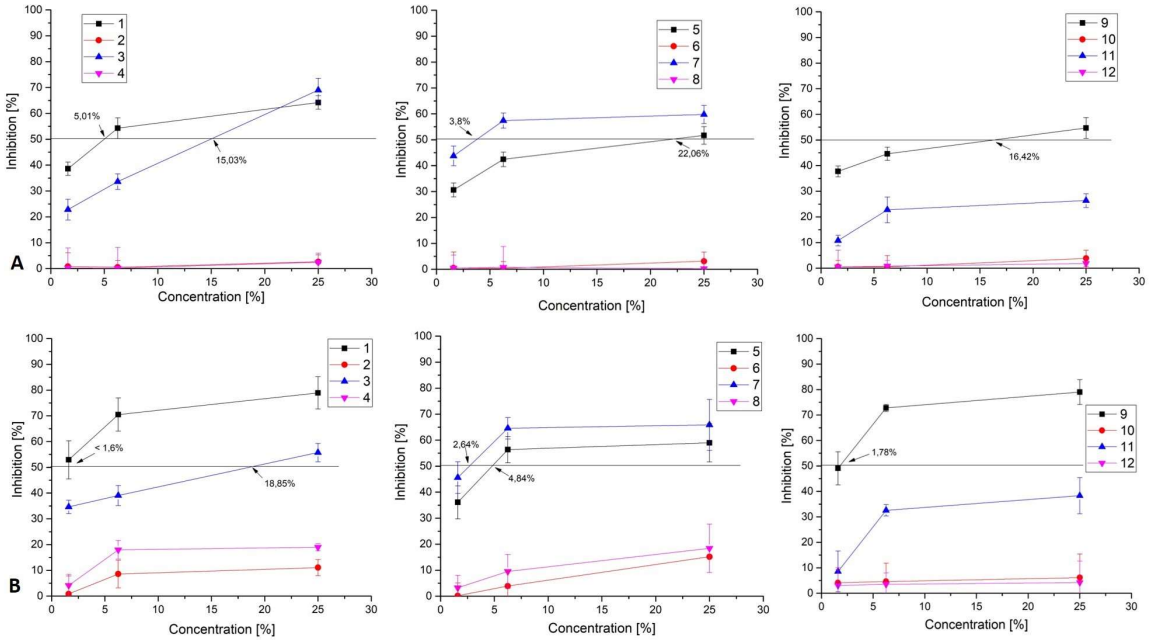


Figure V.7. Antiviral activity of cell-free supernatants of LAB strains 1. KC 5-12, 2. KC 5-13, 3. KC 5-14, 4. KZC 8-21-1, 5. KZC 8-23-5 , 6. C 10-31-3, 7. KO 4-4, 8. KO 3-7-5, 9. KZM 2-11-3, 10. KZM 2-11-1, 11. KZM 2-11 -3 (SM), 12. KZM 2-11-1 (SM) against A) HHV-1 and B) HHV-2

For the cell-free supernatants of the strains that exhibited antiviral activity in replication of both types of viruses, the selective index (SI) was determined based on their cytotoxicity (50% cytotoxic concentration, CC50) and inhibition (50% effective concentration, IC50) against the two virus strains (Table V.6).

The observed effect of antiviral activity can be mainly determined as an effect on the replication cycle of the virus inside the host cells since the results were recorded after one hour of incubation and cannot be related to effects on viral adsorption or penetration (Conti et al., 2009;

Vilhelmova-Ilieva et al., 2022). According to other authors, the dose-dependent antiviral effect is observed with similar products (Mastromarino et al., 2011; Vilhelmova - Ilieva et al., 2022; Möller et al., 2008), stating that it is due to the complex of compounds, produced by LAB.

Table V.6. Determination of selective index of cell-free supernatants of LAB strains that show antiviral activity against HHV-1 and HHV-2

Probe No	CFS of the strains		HHV-1		HHV-2	
		CC_{50}	IC_{50}	SI	IC_{50}	SI
1	KC 5-12	87	5.01	**17.37**	<1.6	**>54**
3	KC 5-14	83	15.03	**5.52**	18.85	**4.40**
5	KZC 8-23-5	>100	22.06	**4.53**	4.84	**20.66**
7	KO 4-4	81	3.8	**21.32**	2.64	**30.68**
9	KZM 2-11-3	85	16.42	**5.18**	1.78	**47.75**

Cell-free supernatants from strains *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3, *L. plantarum* – KO 4-4, KC 5-12, and KZC 8-23-5 were determined to have a selective index greater than 15 against one or both virus models. Although the reported SIs are significantly lower than standard administered products, such as acyclovir with an SI of 560 (determined in parallel analysis by the described method), they may have important practical implications.

Determination of virucidal activity

CFS from two of the studied strains *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3 and *L. plantarum* KC 5-12, which showed the highest activity against HHV-2 virus, were selected to study their virucidal potential against this virus model. After a 6-hour experiment with direct inactivation, both samples showed very weak activity against HHV-2 virions. A difference between the viral control and samples treated with the supernatants was less than 0.5 log (Figure V.8). The determination of the virucidal effect is applied to the assessment of activity during the early stages of the virus life cycle (adsorption or penetration) or directly on the virion and is usually based on the activity of such metabolites as hydrogen peroxide or acids produced by LAB strains (Conti et al., 2009; Khani et al., 2012).

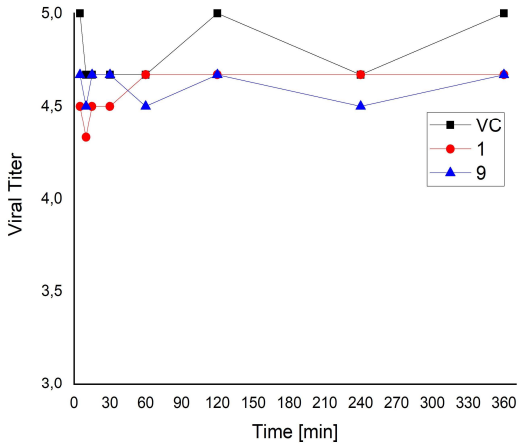


Figure V.8. Virucidal activity of CFS of KC5-12 and KZM2-11-3 against HHV-2

The absence of a virucidal effect in the two selected samples supports the thesis that the established antiviral activity during the replication of the used viral models is determined by other molecular mechanisms and by the complex of biologically active substances produced.

2.5. Determination of the enzyme profile of the studied strains

The appearance of a complex of enzymatic activities in LAB is an important factor in the formation of the characteristics of the food products in which they are included. The specific qualities of fermented food products are determined by the characteristics of the raw materials, the characteristic microbiota, and the applied technology. Strains with different enzyme profiles may contribute to the production of different products (Stojanovski et al., 2013). Glycosidases (α-galactosidase, α-glucosidase, and β-galactosidase) are important enzymes for the fermentation process. β-galactosidase is the enzyme that hydrolyzes the disaccharide lactose to glucose and galactose. β-glucosidases release a wide range of secondary metabolites that improve the taste or aroma of fermented products (Trojanova & Rada 2005; Michlmayr & Kneifel 2014). In this aspect, analyses were carried out to evaluate the main enzyme activities of the studied strains by using the API ZYM test kit, and the obtained results are presented in Table V.7. All tested strains were positive for leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase activity and negative for trypsin, α-chymotrypsin, β-glucuronidase, α-mannosidase and α-fucosidase. Five strains exhibited α-galactosidase activity, and seven - β-glucosidase activity.

Table V.7. Enzyme profile of LAB strains determined with API ZYM kit (bioMérieux, France)

Enzymes	KZM 2-11-1	KZM 2-11-3	KO 3-7-5	KO 4-4	KC 5-12	KC 5-13	KC 5-14	KZC 8-21-1	KZC 8-23-5	C 10-31-3	KBB 7-1	KBB 11
Control	-	-	-	-	-	-	-	-	-	-	-	-
Alkaline Phosphatase	-	-	-	-	1	-	2	1	2	3	1	1
Esterase (C4)	1	2	2	-	1	-	1	2	1	1	1	-
Esterase Lipase (C8)	-	-	1	-	1	-	-	1	-	-	1	-
Lipase (C14)	-	-	-	-	-	1	-	-	-	-	-	-
Leucine arylamidase	3	5	5	5	5	5	5	5	5	5	5	5
Valine arylamidase	1	1	5	3	2	5	3	5	4	5	3	4
Cystine arylamidase	-	-	1	1	1	1	2	4	4	1	3	3
Trypsin	-	-	-	-	-	-	-	-	-	-	-	-
α-chymotrypsin	-	-	-	-	-	-	-	-	-	-	-	-
Acid phosphatase	2	3	3	1	3	3	4	3	4	4	3	3
Naphthol-AS-BI-phosphohydrolase	1	4	1	1	1	1	2	1	4	1	1	2
α-galactosidase	-	-	-	-	-	-	1	-	4	3	2	2
β-galactosidase	5	5	5	5	4	1	5	5	5	1	5	5
β-glucuronidase	-	-	-	-	-	-	-	-	-	-	-	-
α-glucuronidase	-	-	4	-	4	-	5	5	5	-	-	-
β-glucosidase	-	-	-	4	5	-	4	4	5	-	4	5
N-acetyl-β-glucosaminidase	-	-	-	3	4	-	3	3	3	-	2	3
α-mannosidase	-	-	-	-	-	-	-	-	-	-	-	-
α-fucosidase	-	-	-	-	-	-	-	-	-	-	-	-

1, 2, 3, 4, and 5 are the scales for evaluating enzyme activity according to staining intensity; - is for non-determination of enzyme activity.

2.6. Screening for genetic determinants for peptidases in the studied strains

Different protease activities and a complex system of endo- and exopeptidases have been found in different types of LAB. The proteolytic systems of the LAB convert proteins to peptides and amino acids, which are important not only for the development of the bacteria themselves but also for the formation of the aroma and texture of fermented foods. The starter cultures used in the production of fermented milk products have proteolytic activity, allowing their rapid development in milk. During fermentation, milk proteins, mainly casein, undergo proteolytic degradation resulting in bioactive peptides (Kieliszek et al., 2021; Besharati & Lackner 2023). Some of the peptides produced, resulting from the peptidase activity in LAB, exhibit positive health benefits

effects such as antimicrobial, antioxidant, antihypertensive, or immunomodulatory (Kieliszek et al., 2021).

A spectrum of peptidases has been identified in LAB and the genes responsible for their synthesis have been identified (Liu et al., 2010; Qi et al., 2021). To verify the presence of genetic determinants for peptidases in the studied strains, primers were selected for 6 genes found in LAB and associated with the generation of bioactive peptides.

The newly isolated strains were examined for the presence of genetic determinants for the peptidases Aminopeptidase N (pep N), Endopeptidase (pep O), Prolyl aminopeptidase (pep R), X-prolyl dipeptidyl-aminopeptidase (pep X), Tripeptide aminopeptidase (pep T) and Proline dipeptidase (pep Q) (as described in table IV.1) and the results are presented in Table V.8 and Figure V.9.

Table V.8. Presence of genetic determinants for peptidases in the studied strains

Strains	*KZM 2-11-3*	*KZC 8-21-1*	*KO 4-4*	*KC 5-12*	*KZM 2-11-1*	*KZC 8-23-5*	*C 10-31-3*	*KC 5-14*	*KC 5-13*	*KO 3-7-5*
Serial number (Fig. V.9.)	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
Pep N	-	-	+	-	-	-	+	-	-	-
Pap R	-	-	-	-	-	-	+	-	-	-
Pep O	-	-	-	-	-	-	+	-	-	-
Pep T	-	-	-	-	-	-	+	-	-	-
Pep X	-	-	-	-	-	-	+	-	-	-
Pep Q	-	-	-	-	-	-	+	-	-	-

Genes for the investigated peptidases were found at strains *L. sakei* C 10-31-3 for all investigated peptidases and at *L. plantarum* KO 4-4 the gen for pep N was detected. In the rest of the investigated strains, the presence of genes for the 6 peptidases sought was not detected with the used primers.

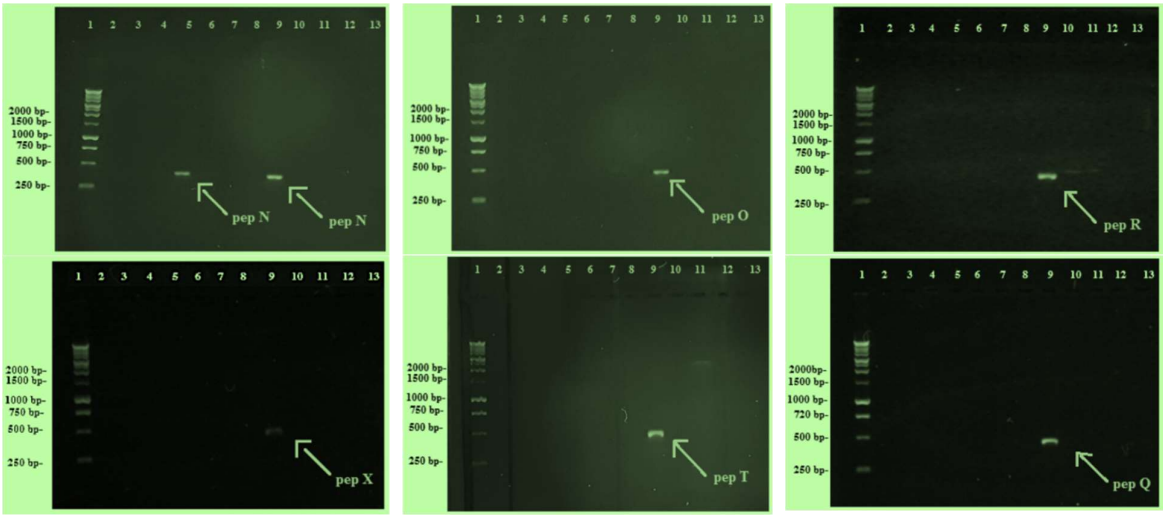


Figure V.9. Peptidase activity of the investigated strains for peptidases pep N, pep O, pep R, pep X, pep T, and pep Q

Peptidase activities in different types of LAB in particular in *lactobacilli* have been the subject of extensive research, including analyses at both physiological and genetic levels. A very wide variety of primers have been designed to detect genes for different peptidases, which in most cases are species-specific (Freiding, S., et al., 2011). The results obtained for the presence of genes

for all six tested peptidases in strain *Latilactibacillus sakei* C 10-31-3 correspond to the results obtained by Freiding, S., et al., (2011) and support the hypothesis of species specificity in the design of screening primers for peptidase genes in LAB.

2.7. Evaluation of the antibiotic resistance of studied strains

Determination of the antibiotic resistance profile

In LAB with applicability in food production and probiotic potential, it is important to investigate resistance to different groups of antibiotic substances. Especially when assessing the probiotic potential of LAB, one of the important criteria is determining the antibiotic resistance profile. On the one hand, due to the possibility of conducting a combination therapy with an antibiotic and a probiotic to restore the normal microflora of the gastrointestinal or urogenital tract, the probiotics must be resistant to the antibiotic with which it will be used in combination, such as combination therapies in diarrhea (Ouwehand et al., 2016). The use of probiotic strains can help inhibit the development of antibiotic-resistant pathogenic strains (Varankovich et al., 2015). On the other hand, it is very important to determine the resistance profile of strains used in fermented foods to reduce the possibility of horizontal transfer of antibiotic resistance genes.

For the determination of the resistance profile of the studied strains, 13 different antibiotic substances were used. The antibiotics used are from the main groups with different mechanisms of action: inhibitors of DNA synthesis - ciprofloxacin, trimethoprim, and rifampicin; cell wall synthesis inhibitors – erythromycin, vancomycin, and ampicillin; protein synthesis inhibitors – chloramphenicol, streptomycin, tetracycline, kanamycin, neomycin, clindamycin and gentamicin (Sharma et al., 2017).

The obtained results are presented in Table V.9 and show that antibiotic sensitivity for the studied antibiotics varies among individual strains, and for some antibiotics, complete resistance was observed for all the studied strains.

Table V.9. Antibiotic resistance profile of newly isolated LAB strains

Antibiotics	µg / disc	KZM 2-11-1	KZM 2-11-3	KO 3-7-5	KO 4-4	KC 5-12	KC 5-13	KC 5-14	KZC 8-21-1	KZC 8-23-5	C 10-31-3	KBB 7-1	KBB 11
Vancomicin (VA)	5	S	I	R	R	R	R	R	R	R	R	R	R
Ampicillin (AMP)	10	SS	SS	SS	I	SS	I	S	SS	I	I	I	I
Streptomycin (S)	10	I	I	R	R	R	R	R	R	R	R	R	R
Tetracycline (TE)	30	SS	SS	S	R	S	R	R	I	R	I	I	I
Chloramphenicol (C)	30	SS	SS	S	R	SS	S	I	I	I	S	I	I
Clindamycin (CD)	2	R	R	SS	R	SS	S	I	I	R	I	I	I
Gentamicin (GEN)	10	R	I	R	R	R	R	R	R	R	R	R	R
Kanamycin (K)	30	R	R	R	R	R	R	R	R	R	R	R	R
Neomycin (N)	30	R	R	R	R	R	R	R	R	R	R	R	R
Trimethoprim (TR)	5	R	R	SS	S	S	R	I	S	S	R	S	S
Erythromycin (E)	15	SS	SS	I	I	I	I	I	I	R	I	I	I
Ciprofloxacin (CIP)	5	R	R	R	R	R	R	R	R	R	R	R	R
Rifampicin (RD)	5	SS	SS	S	I	I	I	S	S	I	R	I	I
Multiantibiotic resistance index (MAR индекс)		**0,46**	**0,39**	**0,46**	**0,69**	**0,46**	**0,62**	**0,54**	**0,46**	**0,69**	**0,62**	**0,46**	**0,46**
According to CLSI, 2020 for the diameter of the zone of inhibition: R – resistant, ≤ 14 mm zone; I – intermediate, 15-19 mm zone; S, SS – susceptible, ≥20 mm zone.													

L. plantarum KO 4-4, *L. plantarum* KZC 8-23-5 and *L. sakei* C 10-31-3 strains have the broadest spectrum of antibiotic resistance. All strains showed complete resistance to kanamycin, neomycin, and ciprofloxacin. All strains tested were susceptible or moderately resistant to ampicillin. Only strain *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-1 was susceptible to vancomycin, and the strain of *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3 has an intermediate susceptibility to vancomycin. Both strains were intermediate to streptomycin. Only one strain was intermediate to gentamicin, *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3. One strain was resistant to erythromycin – *L. plantarum* KZC 8-23-5 and one to rifampicin – *L. sakei* C 10-31-3.

Other studies have shown similar results as all tested LAB strains and *Bifidobacterium* spp. isolated from dairy products were sensitive to clindamycin, ampicillin, penicillin G, erythromycin, and rifampicin, and species-specific differences were observed in susceptibility to chloramphenicol, neomycin, gentamicin, vancomycin, streptomycin, and tetracycline (D'Aimmo et al., 2007). LAB from fermented food products are sensitive to ampicillin and intrinsically resistant to kanamycin and vancomycin, except for strains of the species *L. bulgaricus, L. acidophilus,* and *S. thermophilus*, which are sensitive to vancomycin (Blandino et al., 2008; Nawaz, M. et al., 2011).

Screening of antibiotic resistance genes in the studied strains

One of the important criteria for evaluating technologically relevant and probiotic strains is that they should not carry transferable antibiotic-resistance genes. Several authors indicate that bacteria from the normal microbiota, including *lactobacilli*, can serve as a source for the transfer of genes responsible for antibiotic resistance to various pathogenic microorganisms (Imperial & Ibana 2016). Since LAB can act as a reservoir of transferable antibiotic-resistance genes, it is important to monitor them for their safety (Stefańska et al., 2021). Transferable genetic determinants are common in microbial communities and are observed including in LAB, making these bacteria potential for transfer of these resistance genes. This increases the risk of gene transfer to pathogenic bacteria found both in food matrices and in the GIT (Devirgiliis et al., 2011, Devirgiliis et al., 2013). LAB, which are widely used in the food industry and have acquired resistance, are both obligate homofermentative and obligate and facultative heterofermentative. Some of them are from the genus *Lactobacillus* (*L. helveticus, L. delbrueckii, L. acidophilus*), genus *Limosilactobacillus* (*L. reuteri, L. fermentum*), genus *Lactiplantibacillus* (*L. plantarum*), genus *Lacticaseibacillus* (*L. rhamnosus, L. paracasei*), *Lactococcus lactis, Pediococcus* spp., *Streptococcus thermophilus, Leuconostoc* spp., and *Enterococcus* spp. (Gueimonde et al., 2013; Álvarez-Cisneros & Ponce-Alquicira 2018).

The characterization of the antibiotic resistance profile and the assessment of the transferability of the relevant genes to other bacteria have been analyzed by many researchers. Some authors indicate that antibiotic resistance cannot be transferred from LAB to pathogens such as *Staphylococcus aureus* ssp. *aureus, Staphylococcus haemolyticus, Staphylococcus epidermidis, Listeria monocytogenes, Acinetobacter baumannii, Citrobacter freundii* and *Escherichia coli* (Anisimova and Yarullina, 2018; Guo et al., 2019). Similar studies have shown that the possibility of transferring resistance genes found in LAB to other bacteria is relatively low, making these bacteria safer for various uses, including food production (Anisimova and Yarullina, 2018; Guo et al ., 2019).

The newly isolated LAB strains were tested for the presence of a spectrum of antibiotic resistance genes in the chromosomal DNA and the results are presented in Table V.10. Resistance

genes for certain antibiotics and their primers were selected according to Guo et al., (2019). The genes range in length from 169 bp. to 1429 bp. The results showed that only five of eight vancomycin-resistant strains had a vancomycin resistance gene in their chromosomal DNA. No product was obtained with any of the other primers used for genes associated with antibiotic resistance.

In general, *lactobacilli* show high natural resistance to vancomycin, bacitracin, cefoxitin, metronidazole, nitrofurantoin, and sulfadiazine, as well as to antibiotics that inhibit protein syntheses, such as chloramphenicol, erythromycin, lincomycin, clindamycin, and tetracyclines (Abriouel H., et al., 2015). The two most common antibiotic resistance genes in LAB that can be transferred from and to other microorganisms are tetracycline tet(M) and erythromycin erm(B), followed by the cat genes encoding resistance to chloramphenicol (Moračanin S.V., et al., 2017). However, one of the established mechanisms of multidrug resistance in LAB has been linked to efflux pumps involved in the exclusion of structurally unrelated compounds (Gueimonde M, et al., 2013; Mazurkiewicz P, et al., 2005).

Table V.10. Antibiotic resistance genetic profile of newly isolated strains of LAB

Antibiotics	Primers	*KZM 2-11-1*	*KZM 2-11-3*	*KO 3-7-5*	*KO 4-4*	*KC 5-12*	*KC 5-13*	*KC 5-14*	*KZC 8-21-1*	*KZC 8-23-5*	*C 10-31-3*
VA	vanE	-	-	-	-	-	-	-	-	-	-
	vanX	-	-	-	+	+	-	+	+	+	-
AMP	blaZ	-	-	-	-	-	-	-	-	-	-
	bla	-	-	-	-	-	-	-	-	-	-
	mecA	-	-	-	-	-	-	-	-	-	-
S	aadA	-	-	-	-	-	-	-	-	-	-
	aadE	-	-	-	-	-	-	-	-	-	-
	ant(6)	-	-	-	-	-	-	-	-	-	-
TE	tet(M)	-	-	-	-	-	-	-	-	-	-
	tet(K)	-	-	-	-	-	-	-	-	-	-
	tet(W)	-	-	-	-	-	-	-	-	-	-
C	catA	-	-	-	-	-	-	-	-	-	-
	catA	-	-	-	-	-	-	-	-	-	-
CD	Inu(A)	-	-	-	-	-	-	-	-	-	-
	Inu(B)	-	-	-	-	-	-	-	-	-	-
GEN	aac(6')-aph(2")	-	-	-	-	-	-	-	-	-	-
	aac(6')Ie-aph(2")Ia	-	-	-	-	-	-	-	-	-	-
K	aph(3")-III	-	-	-	-	-	-	-	-	-	-
	ant(2")-I	-	-	-	-	-	-	-	-	-	-
N	aph(3")-I	-	-	-	-	-	-	-	-	-	-
	aph(3")-III	-	-	-	-	-	-	-	-	-	-
TR	dfrA	-	-	-	-	-	-	-	-	-	-
	dfrD	-	-	-	-	-	-	-	-	-	-
E	erm(B)	-	-	-	-	-	-	-	-	-	-
	erm(B-1)	-	-	-	-	-	-	-	-	-	-
	erm(C)	-	-	-	-	-	-	-	-	-	-
CIP	gyrA	-	-	-	-	-	-	-	-	-	-
	parC	-	-	-	-	-	-	-	-	-	-

In the studied strains, multiresistance was observed with a coefficient between 0.39 and 0.69 (table V.9), but the presence of the tet(M), erm(B), and cat genes was not detected. According to EFSA's Qualified Presumption of Safety (QPS), all tested strains belong to species included in the updated list of safe species (https://zenodo.org/records/10534041) with the sole qualification that "they must not contain genes for acquired antimicrobial resistance to clinically relevant antimicrobials'.

2.8. Autoaggregation, coaggregation potential, and hydrophobicity in the studied LAB strains

LAB can form aggregates through the so-called auto-aggregation process (with the same type of bacteria) and through the co-aggregation process (with the participation of different types of bacteria). In the selection of probiotic bacteria, the ability to auto- and/or coaggregation is one

of the significant criteria and is a prerequisite for achieving the desired benefits (Ocaña & Nader-Macias, 2002). Autoaggregation is one mechanism by which LAB protect the host from pathogen colonization by forming a barrier population. Other mechanisms are co-aggregation and adhesion to epithelial cells in the GIT and other organs and systems (Kadyan & Pradhan, 2020).

In this work, all newly isolated LAB were tested for their autoaggregation and coaggregation ability and hydrophobicity. Table V.11 presents the results showing that autoaggregation and hydrophobicity are strain-specific characteristics.

Table V.11. Ability of newly isolated strains for auto-aggregation, co-aggregation and hydrophobicity in percentages

Strains	Auto-aggregation		Co-aggregation	Hydrophobicity
	3h	5h	4h	1h
L. bulgaricus KZM 2-11-1	42.9±8.3	68.0±4.9	13.8±0.8	35.5±5.3
L. bulgaricus KZM 2-11-3	44.2±15.0	70.1±7.3	11.9±4.4	29.7±8.0
L. coryniformis KO 3-7-5	22.6±1.6	44.2±6.1	6.4±1.0	15.6±0.4
L. plantarum KO 4-4	11.3±0.5	17.5±3.0	7.4±0.7	1.2±0.1
L. plantarum KC 5-12	37.3±1.5	60.6±4.4	9.9±0.2	9.9±4.9
P. pentosaceus KC 5-13	17.5±1.9	35.1±3.9	8.7±1.1	16.8±5.8
L. plantarum KC 5-14	19.6±0.3	29.0±5.5	7.8±1.7	29.5±0.7
L. plantarum KZC 8-21-1	16.3±1.4	29.0±7.0	11.5±1.0	5.1±2.6
L. plantarum KZC 8-23-5	12.1±0.4	18.5±1.9	7.2±0.7	6.9±2.8
L. sakei C 10-31-3	21.2±0.2	32.9±3.5	14.4±0.8	2.7±0.0
L. plantarum KBB 7-1	9.0±0.5	11.1±0.1	10.6±0.1	3.2±2.4
L. plantarum KBB 11	16.2±0.7	18.6±0.4	15.9±0.2	0.8±0.6

Data are expressed as a percentage and values are mean of triplicate measurements ± SD. A Pearson correlation (two-tailed) was significant between hydrophobicity and auto-aggregation $p<0,01$.

All isolated strains exhibited autoaggregation of 9 % to 44.2 %, at 3 h incubation at room temperature which increased with time and after 5 h incubation reached 11.1 % to 70.1 %. The best-expressed autoaggregation potential was shown by strains *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-1 (68.0%), *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3 (70.1%), and *L. plantarum* KC 5-12 (60.6%), which defines these strains as interesting candidates with potential for probiotic applications. Good autoaggregation ability in *Lactobacillus* and *Lactiplantibacillus* strains such as *L. plantarum* and *L. delbrueckii* has also been described in other studies (Kadyan & Pradhan, 2020; Darmastuti et al., 2021).

The ability of bacteria to form aggregates with genetically different strains (coaggregation) is discussed as a controversial feature. On the one hand, bacteria should have low co-aggregation abilities to minimize colonization of pathogens in the gastrointestinal tract, and on the other hand, they should have high co-aggregation abilities, as this is one of the possible mechanisms to eliminate pathogens from GIT (Ocaña & Nader-Macias, 2020).

The newly isolated LAB strains were tested for co-aggregation ability with *E. coli* by incubation at room temperature for 4 hours. The strains *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-1 (13.8 %), *L. sakei* C 10-31-1 (14.4 %) and *L. plantarum* KBB 11 (15.9 %) showed the best co-aggregation potential. Other studies described that strains from these genera have different co-aggregation abilities, which is defined as strain-specific (Kadyan & Pradhan, 2020; Janković et al., 2012; Abushelaibi et al., 2017).

Hydrophobicity in probiotic strains is associated with their ability to interact with the epithelial cells of the GIT and, as a consequence, to more effectively exclude pathogens (Narendranath et al., 2001; Simões et al., 2022). Strains with the most pronounced hydrophobicity

were *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-1 (35.5%) and *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3 (29.7%) when incubated at room temperature for 1 hour. Most strains of the *Lactiplantibacillus* genus, except KC 5-14, have shown a relatively low degree of hydrophobicity. Other studies reported similar results for high hydrophobicity in strains of *L. delbrueckii* ssp. *bulgaricus* and low in *L. plantarum* strains (Kadyan & Pradhan, 2020; Ibhaze et al., 2022; Samet & Icen, 2022).

2.9. Evaluation of adhesion abilities of newly isolated LAB strains to mucin (mucoadhesivity)

The mucosal layer found in the GIT protects the epithelial cells from toxins, commensal microorganisms, pathogens, and other environmental irritants. One of the protective mechanisms is based on cellular signaling pathways. Proteins are included in the structure of the mucous membrane, the so-called mucins. Mucins have an important role in physical defense, in the formation of chemical barriers, cell signaling, and in the intestinal regulation of the absorption of elements and immune mediators, such as immunoglobulin-A (IgA) and antimicrobial peptides (Grondin et al., 2020). One of the main criteria for evaluating probiotic properties in candidate probiotic strains is the assessment of their adhesion ability. Concerning this criterion, the adhesion of LAB to mucin is often applied as one of the factors to prove the probiotic properties of bacteria (Mukai et al., 2016).

Proteins that bind to mucin proteins are present on the surface of bacterial cells (Singh et al., 2017). Several studies have evaluated the adhesion abilities of LAB to mucosal proteins, and adhesion and aggregation properties have been established in a strain of *Limosilactobacillus reuteri* (MacKenzie et al., 2010) and in a strain of *Lactococcus lactis* (TIL448), which exhibits high adhesion to porcine gastric mucin (Le et al., 2013). *Lactobacillus fermentum* BCS87 expresses on the cell surface proteins that can bind to mucin (Macıas-Rodrıguez et al., 2009). The presence of *L. delbrueckii* ssp. *bulgaricus* or *Lactobacillus* GG increased two-fold the adhesion of *Bifidobacterium lactis* Bb12 to a mucosal tissue model (Ouwehand et al., 2000), although it was not found that *L. delbrueckii* ssp. *bulgaricus* can bind to mucin.

The adhesion ability of the newly isolated LAB strains to mucin was determined and the results obtained are presented in Figure V.10. The mucoadhesiveness of the newly isolated strains varies and, like the aggregation ability, it can be noted that strain specificity is also observed in this characteristic. The strains *L. coryniformis* KO 3-7-5, *P. pentosaceus* KC 5-13, *L. plantarum* KC 5-14, and *L. plantarum* KZC 8-21-1 have shown the best adhesive ability to mucin with more than 10^5 - 10^6 CFU/ml. Relatively lower adhesiveness was observed in strains *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-1 and *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3 at 10^2-10^3 CFU/mL. Despite the lower mucin binding values reported, these two strains exhibit this specific characteristic not usually described in other strains of this species.

Strains belonging to the species *Lactiplantibacillus plantarum* have shown a different mucin binding ability specific to each strain, with high mucoadhesivity 10^6-10^5 CFU/mL characterized by strains KC 5-14, KZC 8-21-1, KBB 7-1, medium mucoadhesive ability of about 10^4 CFU/mL was reported in strains KC 5-12, KBB 11, and low adhesion ability below 10^4 CFU/mL was determined in strains KO 4-4 and KZC 8-23-5. Other authors also found that strains of the species *L. coryniformis* and *L. plantarum* exhibited well-defined adhesive properties (Singh et al., 2-17; Jatmiko et al., 2017). No literature review found evidence of *Latilactobacillus sakei* strains indicating mucoadhesivity, but the results obtained in the present study show that strain *L. sakei* C 10-31-3 had a high mucin binding ability above 10^5 to 10^6 CFU/mL.

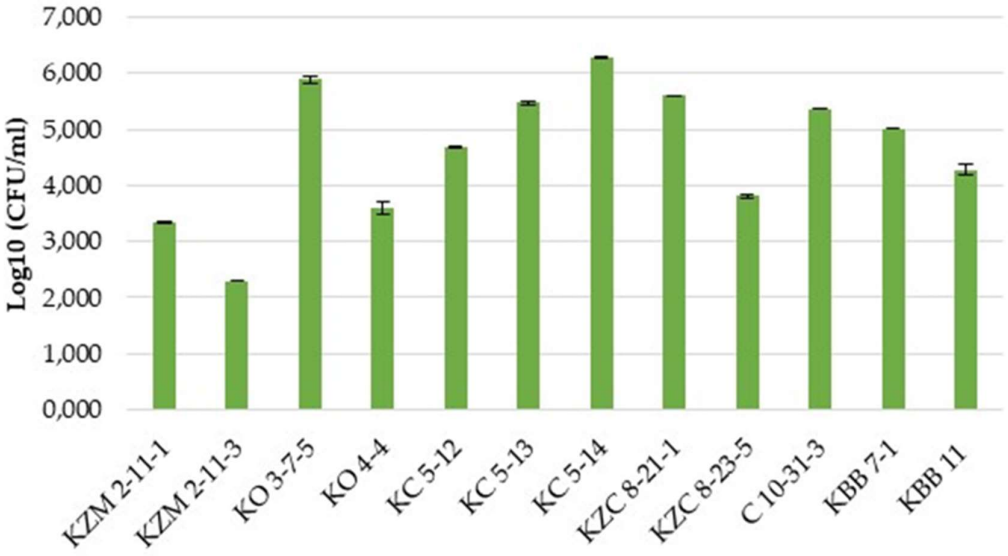


Figure V.10. Adhesive ability of LAB isolates to mucin
Data are presented as Log10 (CFU/ml) ± SD.

The results obtained for the determination of aggregation abilities, hydrophobicity (Table V.11), and adhesive abilities to mucin (Fig. V.10) were compared to establish a correlation between them using Pearson's correlation (two-sided). A positive correlation was found between autoaggregation and hydrophobicity at the $p < 0.01$ level. Other studies have shown different correlations using Spearman's correlation coefficient in *Lactobacillus* strains, from which it is claimed that their adhesion ability has a positive correlation with autoaggregation and hydrophobicity (Valeriano et al., 2014), and the autoaggregation abilities of *lactobacilli* are highly correlated with their coaggregation potential with different pathogens (García-Cayuela et al., 2014).

2.10. Determination in vitro of studied LAB strains' abilities to survive in simulated conditions of different GIT departments

In vitro evaluation of the growth of newly isolated strains under stress factors of different GIT departments

The low pH in the stomach and the presence of gastric enzymes and bile salts are the main barriers of the GIT, through which the probiotic bacteria must pass and maintain their viability and activity to exert their beneficial effects in the lower compartments of the GIT of the host (Menconi et al., 2014). The time for the passage of the food together with the microbiota through the stomach is from 15 minutes to 4 hours, during which the cells of the bacteria are exposed to aggressive conditions, such as pH around 1.5 - 2, and the presence of enzymes, such as pepsin. Pepsin is one of the main digestive enzymes that breaks down proteins into peptides and is active at a low pH between 1.5-2. After the stomach, the food passes to the small intestine, where the pH of pancreatic secretions is 7.8 - 8.4, and then to the large intestine. The time for food to pass through the intestines is about 12-24 hours, as pancreatic enzymes break down the various components (Sensoy I., 2021). Bile secretion is secreted in the duodenum and also participates in the digestive processes, mainly of lipids. Bile salts from bile secretion can be used by gut bacteria as an environmental signal or in some cases as electron receptors or as nutrients. But bile salts can also

disrupt bacterial membranes, denature proteins, and cause DNA damage (Urdaneta & Casadesús, 2017).

To evaluate the ability of the studied strains to stretch under stressors, they were cultured under the influence of various factors characteristic of the upper and lower sections of the GIT, and inhibition coefficients were determined. The results are presented in Table V.12, with the strains showing different inhibition coefficients under acidic conditions and in the presence of pepsin.

Table V.12. Coefficient of inhibition of newly isolated strains in acidic conditions, pancreatic enzymes, and bile salts

Coefficient of inhibition	**3h**	**24h**		**24h**	
Strains	**Pepsin (1 mg/ml) +pH2**	**Pancreatin (1 mg/ml)**	**Bile salts 0.1%**	**Bile salts 0.3%**	**Bile salts 1.0%**
L. bulgaricus KZM 2-11-1	0.70±0.19	0.17±0.12	0.72±0.33	1.09±0.09	1.12±0.08
L. bulgaricus KZM 2-11-3	0.60±0.23	-1.12±0.36	1.04±0.06	1.26±0.04	1.20±0.08
L. coryniformis KO 3-7-5	0.97±0.03	0.17±0.05	0.68±0.04	1.04±0.07	1.11±0.06
L. plantarum KO 4-4	0.93±0.59	-0.04±0.02	0.10±0.06	0.69±0.09	0.94±0.37
L. plantarum KC 5-12	0.93±0.32	-0.01±0.01	-0.05±0.29	0.31±0.13	0.45±0.30
P. pentosaceus KC 5-13	1.01±0.05	0.001±0.07	0.14±0.09	0.18±0.05	0.69±0.06
L. plantarum KC 5-14	1.02±0.12	0.51±0.02	0.61±0.05	0.60±0.04	0.52±0.02
L. plantarum KZC 8-21-1	0.97±0.29	0.20±0.04	0.11±0.01	0.58±0.13	0.81±0.49
L. plantarum KZC 8-23-5	0.94±0.05	0.07±0.01	0.11±0.04	0.39±0.11	0.85±0.31
L. sakei C 10-31-3	1.20±0.12	-0.26±0.18	0.46±0.09	1.04±0.04	1.06±0.04
L. plantarum KBB 7-1	1.20±0.11	0.16±0.01	0.43±0.04	0.95±0.03	1.04±0.07
L. plantarum KBB 11	1.02±0.03	0.26±0.05	0.50±0.06	0.83±0.02	0.97±0.04

The values of the coefficient of inhibition are mean values of triplicate measurements ± SD.

In the strains *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-1 and *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3 the lowest coefficient of inhibition was determined in the presence of pepsin and pH 2. The presence of pancreatic enzymes had no effect or very little inhibition was observed in all strains. Under the influence of bile salts in different concentrations (0.1%, 0.3%, 1.0%), it was observed that as the concentration increased, the coefficient of inhibition determined for the different strains also increased. At a bile salt concentration of 0.1%, a very low coefficient of inhibition was determined for five strains (KO 4-4, KC 5-12, KC 5-13, KZC 8-21-1, and KZC 8-23-5). At a concentration of 0.3% bile salts, in only three strains (KC 5-12, KC 5-13, and KZC 8-23-5) the coefficient of inhibition was less than 0.4, while at concentrations of 1.0% in only one strain KC 5-12 the coefficient of inhibition was close to 0.4. Strains in which the coefficient of inhibition is up to 0.4 at a bile salt concentration of 0.3% can be considered suitable probiotic candidates according to Salehizadeh et al., (2020).

LAB are acidophilic and tolerant to low pH, however high free (H+) concentration can cause growth inhibition (Menconi et al., 2014). Changes in the external environment such as the increased concentration of bile salts and pH can cause metabolic disturbances leading to even cell death. The presence of a constant stress factor in microorganisms is a prerequisite for developing resistance mechanisms for survival in such conditions.

Intracellular pH decreases as the acidity of the environment increases, resulting in energy consumption by cells to keep their metabolic activity under control. When pH drops too low, pH homeostasis is disrupted, causing protein and DNA damage and structural rearrangements in cells (Urdaneta & Casadesús, 2017; Guan & Liu, 2020). To determine whether exposure to stress factors

affects the ability of cells to grow and cope with metabolic changes, the growth of the studied strains was evaluated by measuring the optical density (OD) of the bacterial culture at 600 nm and according to Missotten et al., (2009) results are presented as: 1) "+", growth ≤ 0.2 OD; 2) "++", 0.2 < growth ≤ 0.5 OD; 3) "+++", growth > 0.5 OD (table V.13).

Table V.13. Growth rate of bacteria in low pH and pepsin, pancreatic enzymes, and bile salts

Strains	Growth rate				
	Pepsin (1 mg/ml) +pH2	**Pancreatin (1 mg/ml)**	**Bile salts 0.1%**	**Bile sats 0.3%**	**Bile sats 1.0%**
L. bulgaricus KZM 2-11-1	+	++	+	n.d.	n.d.
L. bulgaricus KZM 2-11-3	+	+++	n.d.	n.d.	n.d.
L. coryniformis KO 3-7-5	n.d.	+++	++	n.d.	n.d.
L. plantarum KO 4-4	n.d.	+++	+++	++	+
L. plantarum KC 5-12	+	+++	+++	+++	+++
P. pentosaceus KC 5-13	n.d.	+++	+++	+++	+++
L. plantarum KC 5-14	n.d.	+++	+++	+++	+++
L. plantarum KZC 8-21-1	+	+++	+++	+++	++
L. plantarum KZC 8-23-5	+	+++	+++	+++	++
L. sakei C 10-31-3	n.d.	+++	+++	n.d.	n.d.
L. plantarum KBB 7-1	n.d.	+++	+++	+	n.d.
L. plantarum KBB 11	n.d.	+++	+++	++	+

Growth of the bacteria: +, growth ≤ 0,2; ++, 0,2 < growth ≤ 0,5; +++, growth > 0,5. n.d.- no growth detected.

The results have shown that some of the strains could retain the ability to grow at pH 2 and in the presence of pepsin, all strains have shown very good growth in the presence of pancreatin. At different concentrations of bile salts, changes were observed in terms of bacterial growth, even a lack of growth in some of the strains, reported as a lack of change in the measured optical density (Table V.13).

Evaluation of the survival of the studied strains under the direct impact of stress factors characteristic of different departments of the GIT

Most microorganisms can adapt to changes in external conditions to survive under these conditions and have the opportunity to develop and multiply. For example, to adapt microorganisms to acidic environments, they develop improved acid tolerance through various metabolic regulatory mechanisms. Microorganisms can protect or repair macromolecules such as DNA and proteins through specific proteins (DNA polymerase, DNA ligase, chaperones) that are induced, for example, under acid stress. Microorganisms can improve metabolism and redox factors for survival and growth by regulating the glycolytic pathway. The response to stressors can involve different elements of cellular metabolism and be species, even strain-specific (Guan & Liu, 2020).

To assess viability, newly isolated LAB strains were directly exposed to stressors characteristic of GIT by incubation with pepsin at low pH and with bile salts at a concentration of 0.3%. The survival results for the tested strains as log10 (CFU/ml) and in percentages are presented in Table V.14. In the presence of pepsin and low pH, survival above 50% was observed for most strains. In the strains, *L. bulgaricus* KZM 2-11-1, and *L. bulgaricus* KZM 2-11-3, no ability to survive under the direct influence of pepsin and pH 2 within 3 hours was reported. Strain *L. sakei* C 10-31-3 was also not reported to survive in such conditions. In the presence of bile salts at a concentration of 0.3%, survival of over 45% to 94% was reported for all strains. Other studies have reported that strains of the species *L. plantarum* and *P. pentosaceus* have the best survival

ability under the effects of different factors in GIT (Singh et al., 2017; Pinto et al., 2020; Sharifi Yazdi et al. al., 2017; Salehizadeh et al., 2020).

Table V.14. Survival capability of LAB strains in acidic conditions and bile salt

Strains	pH of strains culture	Pepsin (1 mg/ml) +pH2			Bile sats 0.3%		
		Control 3ч.	Pepsin+ pH2 3ч.	Survival (%)	Control 24ч.	Bile salts 24ч.	Survival (%)
L. bulgaricus KZM 2-11-1	3.48	7.73±0.05	n.d.	-	5.50±0.01	2.48±0.35	45
L. bulgaricus KZM 2-11-3	3.50	8.13±0.02	n.d.	-	4.40±0.09	2.56±0.18	58
L. coryniformis KO 3-7-5	3.52	8.25±0.04	5.13±0.01	62	7.91±0.04	4.21±0.00	53
L. plantarum KO 4-4	3.02	8.84±0.03	8.05±0.01	91	8.02±0.02	6.94±0.00	87
L. plantarum KC 5-12	3.19	7.53±0.05	6.09±0.01	81	8.30±0.16	6.91±0.00	83
P. pentosaceus KC 5-13	3.20	8.71±0.01	4.31±0.02	50	8.18±0.06	7.24±0.01	88
L. plantarum KC 5-14	3.03	8.64±0.04	8.09±0.01	94	8.81±0.03	5.74±0.04	65
L. plantarum KZC 8-21-1	2.91	8.33±0.01	7.95±0.00	95	8.39±0.01	6.56±0.07	78
L. plantarum KZC 8-23-5	2.96	8.72±0.02	8.34±0.00	96	8.81±0.10	6.73±0.01	76
L. sakei C 10-31-3	3.84	8.15±0.03	n.d.	-	7.56±0.03	7.12±0.00	94
L. plantarum KBB 7-1	3.84	8.89±0.02	6.22±0.08	70	8.44±0.01	7.13±0.01	84
L. plantarum KBB 11	3.81	8.83±0.01	6.59±0.05	75	8.15±0.07	7.04±0.02	86

The values of log10 (CFU/ml) are means of measurements ± SD. Survivability is expressed as % of mean values. n.d.- no growth detected.

To determine with statistical significance whether there was a relationship between the ability of the test strains to survive in acidic conditions and in the presence of bile salts and the pH level at which each strain was cultured, a Pearson correlation analysis was performed. A positive correlation was found between culture pH level and survival in the presence of pepsin and low pH, with a Pearson correlation coefficient of significance $p<0.01$. This indicates that strains that produce higher amounts of acids (mainly lactic acid) and a lower pH observed when they are cultured can more successfully survive under acidic conditions. The relative tolerance to acidic conditions is strain-specific and the survival of the particular strain is related to the control mechanisms of intracellular pH and H +, and ATPases play a major role (Hutkins & Nannen, 1993). This indicates that strains that reduce extracellular pH are better able to survive the acidic conditions of the stomach, as a result of more complex mechanisms to protect and repair their cells under such conditions.

2.11. Evaluation of the probiotic potential of the newly isolated LAB strains

To make a comparison and quantitative assessment of the probiotic potential of the studied strains, a scoring system was applied for each strain for the studied properties and determination in percentage of the maximum possible sum as probiotic potential (Gökmen, G.G., et al., 2024). Table V.15 presents the evaluation points for the individual investigated properties, and the probiotic potential in percentages is presented in Figure V.11.

In the evaluation of the probiotic potential of the studied strains, it was determined that the best-expressed potential was reported at the strain *L. plantarum* KC 5-12, in which, based on the main characteristics, a potential of 80% was determined. The next two strains KC 5-14 and KZC 8-21-1 with a probiotic potential score above 70% are also of the *L. plantarum* species. At the strain, *P. pentosaceus* KC 5-13, over 70% probiotic potential was also reported. It is important to note that in both strains *L. bulgaricus* KZM 2-11-1 and *L. bulgaricus* KZM 2-11-3 a significant probiotic potential was also determined, which in strain KZM 2-11-3 was 60% for the main characteristics and over 60% with the additional characteristics. Strains of the species *L. bulgaricus* are traditionally part of the starter cultures for the production of fermented dairy

products, and the two strains of this species that are included in the present study are isolates from traditionally prepared yogurt. Similar strains with probiotic potential are of interest for inclusion in production starter cultures, for increasing the functional characteristics of the final products.

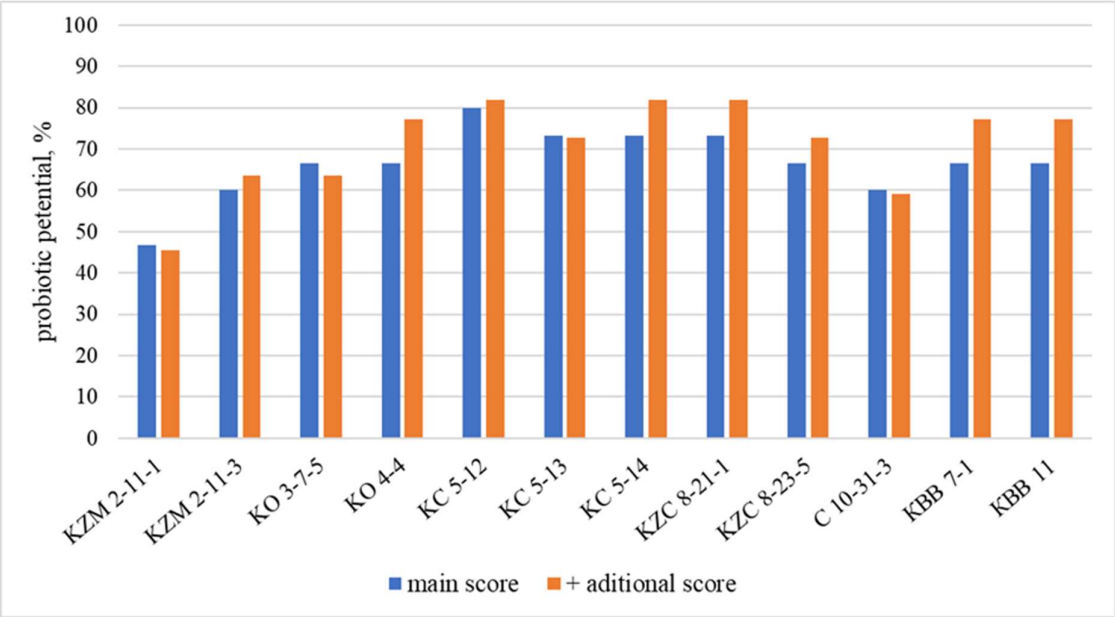


Figure V.11. Probiotic potential of studied LAB strains in percentage (%)

Table V.15. Quantitative evaluation of the probiotic potential of the studied strains according to a score system

Probiotic and functional characteristics			**Evaluation of the studied strains**											
	evaluation		**KZM 2-11-1**	**KZM 2-11-3**	**KO 3-7-5**	**KO 4-4**	**KC 5-12**	**KC 5-13**	**KC 5-14**	**KZC 8-21-1**	**KZC 8-23-5**	**C 10-31-3**	**KBB 7-1**	**KBB 11**
	indicator	***points***												
Activity against bacterial and yeast test pathogens	No activity	0	2	2	2	3	2	2	2	2	2	2	3	3
	until 3 test MO	2												
	More than 3 test MO	3												
Antiviral activity	No activity	0	0	2	0	2	2	0	1	0	2	0	0	0
	SI index ≤ 15	1												
	SI index > 15	2												
MAR index	< 0.2	0	1	1	1	1	1	1	1	1	1	1	1	1
	≥ 0,2	1												
Autoaggregation potential	< 20	0	1	1	1	0	1	1	1	1	0	1	0	0
	≥ 20	1												
Coaggregation	none	0	2	2	1	1	1	1	1	2	1	2	2	2
	< 10%	1												
	≥ 10	2												
Mucoadhesivity	none	0	1	1	3	1	2	3	3	3	1	3	2	2
	Under 10^4 CFU/ml	1												
	10^4 - 10^5	2												
	Above 10^5	3												
Survival under simulated conditions of GIT	Pepsin + pH2		0	0	2	2	2	2	2	2	2	0	2	2
	No survival	0												
	Until 40 %	1												
	Above 40 %	2												
Coefficient of inhibition at 0.3% bile salts	Until 0,4	1	0	0	0	0	1	1	0	0	1	0	0	0
	Above 0,4	0												
SCORE		**Max 15**	7	9	10	10	12	11	11	11	10	9	10	10
Additional properties														
Inhibition food-associated contaminants	Molds 100% inhibition		2	2	3	4	3	3	4	4	3	2	4	4
	A. niger	1												
	F.proliferatum	1												
	P. claviforme	1												
	A. flavus	1												
	Yeasts more than 48 h		1	3	1	3	3	2	3	3	3	2	3	3
	K. lactis	1												
	K. marxianus	1												
	S. cerevisiae	1												
TOTAL SCORE		**Max 22**	10	14	14	17	18	16	18	18	16	13	17	17

3. Determination of the main technological characteristics of the investigated strains

3.1. Determination of total titratable acidity

Organic acids, such as lactic, acetic, etc., are one of the main metabolites produced by LAB during fermentation. The organic acids produced can improve the taste of fermented foods and prevent spoilage, resulting in better consumer acceptance. The most important characteristics of fermented foods that are determined by the organic acids produced are related to taste and aroma, as well as antimicrobial and antioxidant effects (de Souza, E.L., et al., 2023). This is the main reason why one of the main technological characteristics in the selection of LAB strains is related to determining their acid-forming capacity.

To evaluate the acid-forming capacity of the studied strains, they were tested for total titratable acidity (TA) when cultured on MRS broth medium and SM medium (Figure V.12). Total titratable acidity in all tested strains was lower in SM medium compared to MRS broth medium. The strains with the lowest titratable acidity in SM medium were *L. sakei* C 10-31-3 and *P. pentosaceus* KC 5-13, and in MRS broth medium were *L. coryniformis* KO 3-7-5 and *L. sakei* C 10-31-3. In both strains, *L. delbrueckii* ssp. *bulgaricus* and in strain *L. plantarum* KBB 7-1 the TA was up to 150 °T during 24 hours of cultivation on medium 10% SM, and in the rest of the studied strains, this indicator was lower.

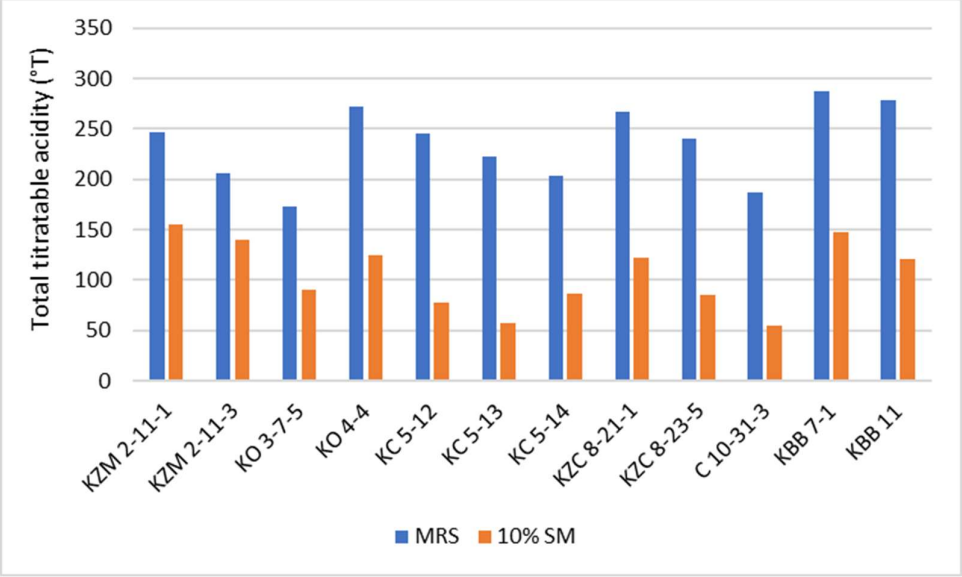


Figure V.12. Total titratable acidity of the studied LAB strains when they were cultured on medium MRS broth and medium SM, (°T)

3.2. Durability in technological processes - determined by survival during the freeze-drying process and selection of suitable protective media.

Freeze-drying (lyophilization) is one of the main technological stages that are part of the production of starter cultures and probiotic preparations. Establishing good technological stability and suitable conditions for carrying out basic processes while preserving the vitality and activity of potentially significant LAB strains are an important stage of their overall evaluation for inclusion in new products.

In newly isolated LAB strains, a study was carried out to evaluate survival during the freeze-drying process in two types of protective media and after a period of storage at 4°C. Using fresh medium 10% skimmed milk as the most cost-effective possible lyoprotectant, it was found

that some of the strains showed a significant reduction in CFU, both immediately after the freeze-drying process and during the storage period. In both strains, *L. delbrueckii* ssp. *bulgaricus* a more significant reduction in CFU was observed, with up to 1 log after process and up to 3 log over a 6-month storage period. Up to 1 log reduction in CFU was observed in the other tested strains (Figure V.13).

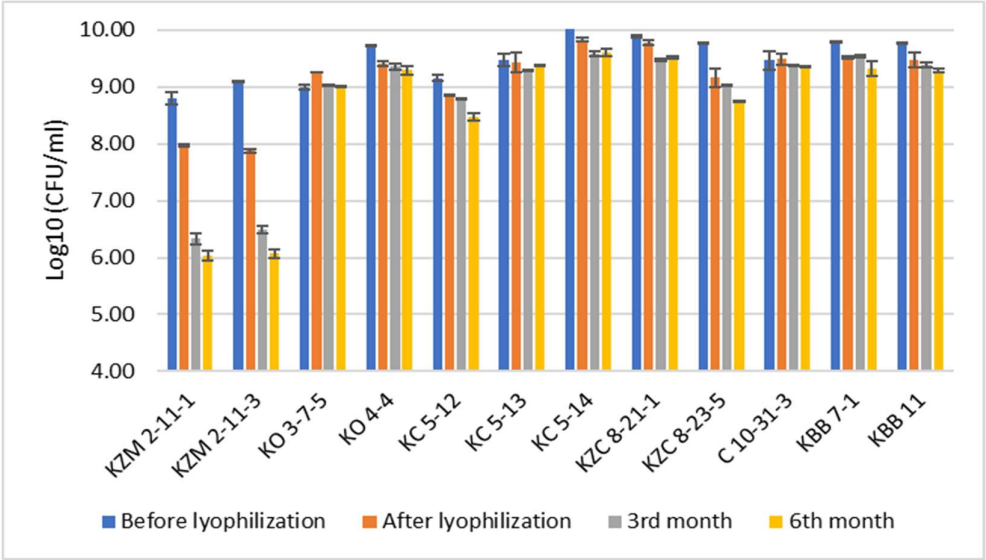


Figure V.13. Survival of the studied strains during the freeze-drying process and storage time in the protective medium of 10% skimmed milk

The obtained results, led to a need for a more suitable lyoprotective medium. As a result of a study, in the scientific literature (Teng et al., 2017; Stefanello et al., 2018; Gul et al., 2020), was used 10% skim milk, including 5% lactose and 2% ascorbic acid (vitamin C). Significantly better survival was observed with this variant of protector medium, including both strains KZM 2-11-1 and KZM 2-11-3, which maintained the order of 10^7 to 10^8 CFU over the storage period, which determined this protected environment as more suitable for application (Figure V.14).

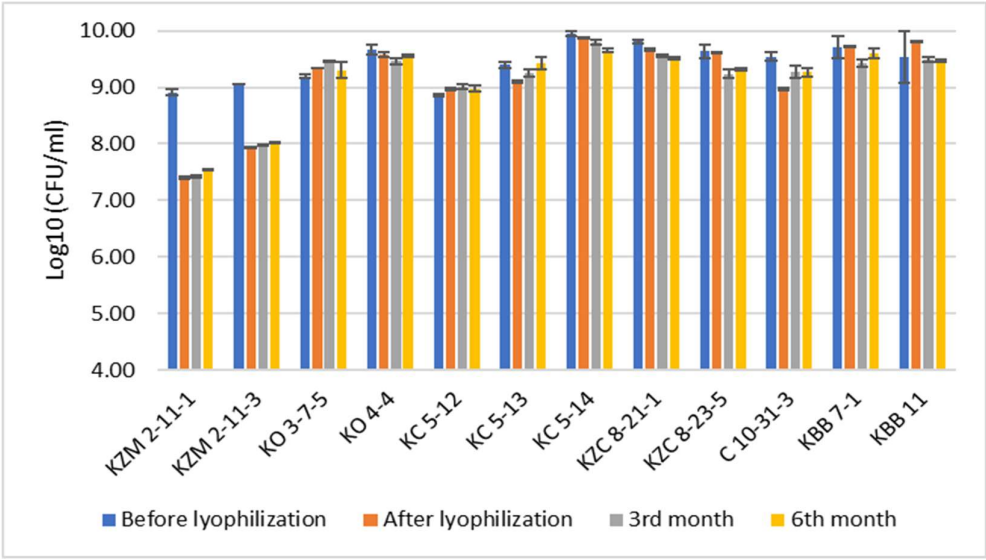


Figure V.14. Survival of the studied strains during the freeze-drying process and storage time in the protective medium of 10% skimmed milk + 5% lactose + 2% vitamin C

The obtained results on the influence of the component composition of the protective media during the freeze-drying process are a very good basis for developing a technological solution for obtaining in dried form and storage while preserving the viability and activity of potentially probiotic and/or technologically significant LAB strains.

4. Incorporation of selected strains from the new LAB isolates into a model product and determination of main characteristics

4.1. Selection of strains and obtaining a model product

For the purposes of subsequent experimental work, two strains were selected from the group of new isolates, *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3, *Lactiplantibacillus plantarum* KC 5-12. Strain KC 5-12 was selected because of the determined highest probiotic potential of the group of tested strains, which it is a cow's cheese isolate. Strain KZM 2-11-3 was selected due to its belonging to the species *L. delbrueckii* ssp. *bulgaricus*, which is necessarily present in symbiont starter cultures for yogurt, but also shows significant probiotic potential for strains of this type (Figure V.11), exhibiting well-expressed aggregation properties, mucoadhesiveness, antiviral activity and ability to inhibit food-associated contaminants. The selected strains were included in the production of model products - yogurt, alone and in combination, and as control variants, yogurt samples were prepared with a commercial starter culture used for the production of yogurt according to BDS 12:2010. A detailed description of the four variants of prepared yogurt samples is described in point 12, section Materials and Methods

4.2. Study of the main physicochemical characteristics of the four variants of the model product during the fermentation process and during the storage period

Each food product has relevant standards and certain quality requirements that must be met. Some of the main indicators that are monitored during the fermentation process to evaluate the quality of the product are pH, redox potential (RP), and titratable acidity (TA) (Lee W. & Lucey J., 2010; Martin et al., 2011; Garcia & Cisneros, 2013). In the present study, the characteristics of the four types of yogurts were determined by analyzing various parameters during the fermentation process and during a storage period of up to 28 days. Figure V.15 shows a picture of the 4 yogurt variants prepared as described in the section Materials and Methods.

Figure V.15. Samples of yogurt in 0 days of storage at 4°C

Monitoring of pH, redox potential, and titratable acidity in the four yogurt variants

Figure V.16 presents the results of monitoring the dynamics of the three main parameters pH, RP, and TA during the fermentation process and during the storage period at 4 °C for four types of yogurt samples. The development of each of the selected strains in the prepared samples leads to the accumulation of lactic acid and other organic acids, as a result of which the nutrient matrix is acidified, the pH gradually decreases, and the concentration of hydrogen ions (H+)

increases (Ito et al., 2003). Lactic acid production is an important mechanism for the characteristics of the final yogurt product by determining the decrease in pH and the subsequent acid coagulation of milk proteins to form the gel structure of yogurt (Kwasi Kpodo et al., 2014).

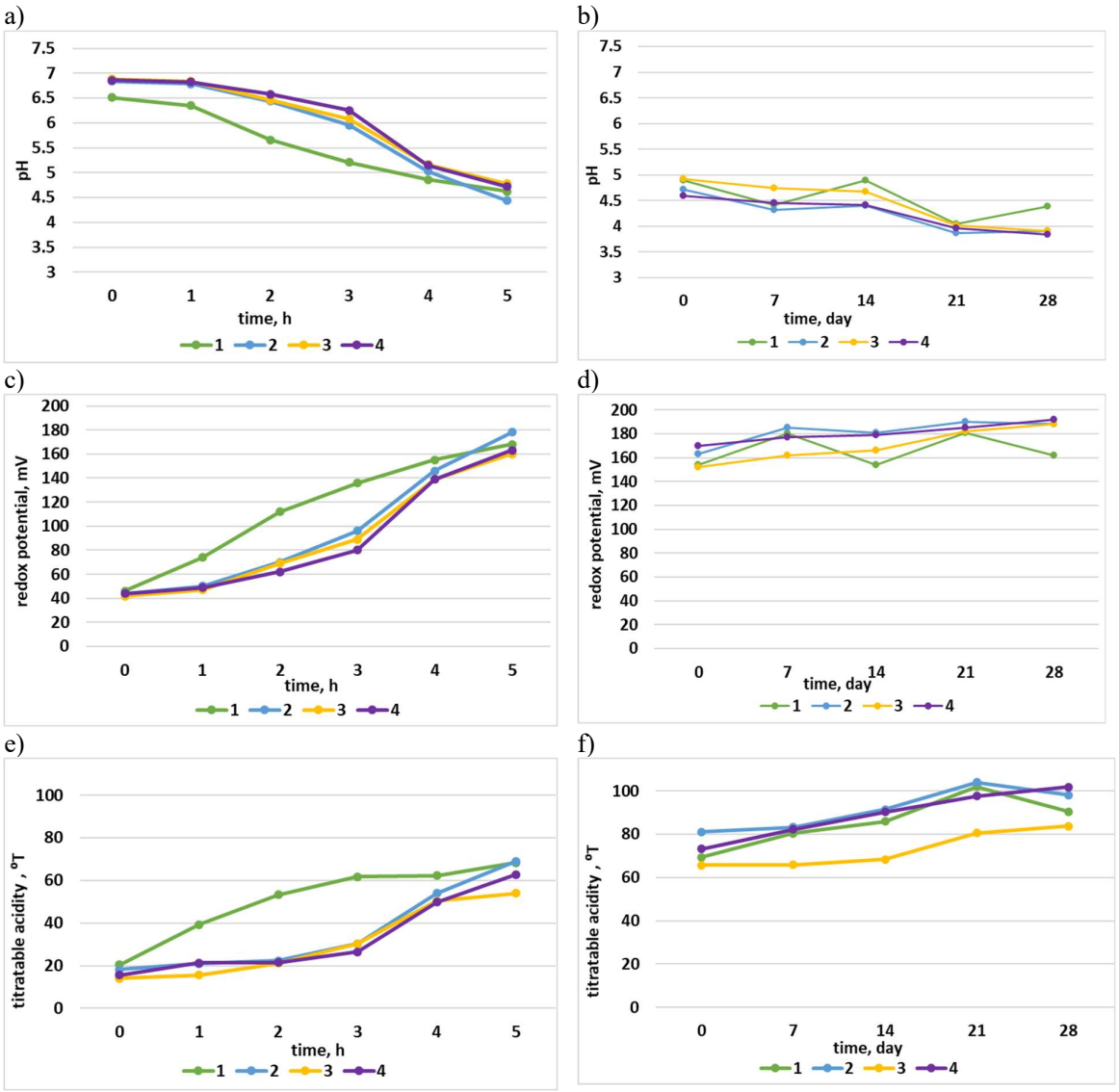


Figure V.16. Parameters of yogurt a) pH, c) RP, and e) TA during the fermentation process and b) pH, d) RP, and f) TA during storage time

During the fermentation process to obtain the yogurt model products, two stages are observed (Figure V.16). During the first stage, up to about the 4th hour, it is noticed that the curves of change of the measured pH and TA parameters, in the samples of yogurt with two strains included separately or in combination, continue to differ from those of the control sample of yogurt (option 1). The second stage includes the last hour of the fermentation process, where the curves of the measured parameters in all samples are similar and the pH at the end of the fermentation process in all yogurt samples is similar to that in the control variant and is within 4.4 -4.8. The pH curves of the yogurt samples were similar for the storage time and the pH was in the range of 3.8–4.8 on day 28.

Redox potential (RP) is one of the most important parameters in fermentation processes. According to Martin et al., (2011), the change in RP stimulates the production of aromatic

compounds that determine the characteristics of the final products. TA is also an important parameter related to the quality of the final product. For both parameters, RP and TA no large variations were observed compared to the control variant, at the fermentation stage and during storage time. At the end of the fermentation process, the RP was in the range of 160–180 (mV), and during storage, it varied slightly in the range of 150–190 (mV). TA at the end of the fermentation process was similar to that of the control variant, 50–70 (°T). Slight variations were observed in TA during storage time, and in comparison with the control variant, in sample 3 the TA remained the lowest, and in samples 2 and 4 the values were similar to the control variant. Similar results for the three main physicochemical parameters of yogurt during the fermentation process have also been described by other authors (Parvarei et al., 2021) and such variations in parameters during storage have also been reported in similar experiments (Fayyaz et al., 2020).

Determination of water holding capacity (WHC) and syneresis during the storage time of the four yogurt variants

The main components in the composition of yogurt are water, proteins, fats, and polysaccharides, and it is also a rich source of vitamin B, calcium, magnesium, etc. (Lee W. & Lucey J., 2010; Hadjimbei et al., 2022). The decrease in pH during fermentation and denaturation of milk proteins leads to the formation of the gel-like structure in yogurt. The hydrophilic part of the casein molecule has the ability to bind with water molecules and this protein-water interaction affects the color, structure, emulsification, and sensory properties of the final product. The water content of the product depends on the composition of the milk, the amino acid composition of the proteins, their molecular weight, the properties of the constituent amino acids, and other environmental factors such as pH, temperature, ionic strength, and the species composition of the bacterial starter cultures. The water content of the product is essential for microbial growth (Hole, 2003; Hayes et al., 2007; Mok et al., 2008; Haque et al., 2017). Zayas, (1997) defined water holding capacity (WHC) of foods as the ability to retain their own and added water during the application of forces, ie. pressing, centrifuging, or heating. The WHC of the protein gel in yogurt is a very important characteristic related to viscosity and syneresis (Zayas, 1997). This parameter in yogurt affects the texture of the product and is an important parameter in quality research.

The WHC results of the yogurt samples are presented in Table V.16. The sample with the highest WHC in the casein micellar structure, similar to the yogurt control, was variant 3, although no significant differences were reported between individual samples. No statistically significant difference was observed in WHC during storage of the yogurt samples. Studies by other authors also show that the values for WHC percentages are approximately similar to those obtained in this experiment (Dan et al., 2012; Parvarei et al., 2021).

Table V.16. Water holding capacity (WHC) of four variants of yogurt

	Water holding capacity (%)			
	Samples of yogurt			
Storage time	**1**	**2**	**3**	**4**
0 day	36.7±0.02	35.1±2.60	37.7±3.37	35.5±0.20
7 days	35.8±0.96	33.3±0.13	35.3±0.74	34.3±0.12
14 days	38.2±0.92	36.5±1.26	36.7±0.96	35.8±1.15
21 days	36.4±0.89	34.3±0.24	34.9±0.94	34.3±0.52
28 days	36.7±2.44	35.1±2.17	37.7±2.13	35.5±2.11

Percentages of water holding capacity were expressed as means ± SD. One-way analysis of variance (ANOVA) using Tukey's test was applied to compare mean values of yogurt samples during storage time ($p > 0,05$).

The structural instability of foods is illustrated by the release of water that is lost from gels, in the case of yogurt gel, especially during low-temperature storage (Hole, 2003). The matrix of casein micelles in yogurt can be restructured for various reasons, including as a result of the metabolic activity of the starter culture strains, acidification, and processing after the fermentation step, resulting in the separation of an aqueous phase. Syneresis is a phenomenon of loss of water content and is undesirable in yogurt. Syneresis is considered a yogurt texture defect (Playne et al., 2003).

The results of determining the syneresis in the tested yogurt variants are presented in Table V.17, and in general, a slight decrease in the syneresis was observed from the first day to the 28th day of product storage, and this indicator slightly varied in the different periods of storage time. The yogurt samples with the lowest amount of water separated, i.e. similar to the control yogurt, were samples 3 and 4. After the first 7 days of storage, the lowest syneresis was reported for sample 4, and on day 14, the lowest values were for sample 3. After statistical analysis of the data of syneresis, no significant differences were observed between samples compared to the control variant. Similar results of syneresis have been found in other studies (Benezech T. & Maingonnat J.F., 1994; Soni et al., 2020).

Table V.17. Syneresis of the yogurt samples

	Syneresis (%)			
	Samples of yogurt			
Storage time	**1**	**2**	**3**	**4**
0 day	8.6±1.50	12.6±0.50	10.0±3.25	9.4±0.80
7 days	7.6±0.80	12.9±2.70	10.2±0.70	7.8±0.68
14 days	7.3±0.95	10.0±0.30	8.4±0.00	9.6±1.44
21 days	8.6±1.80	8.8±1.10	8.3±1.20	10.3±0.78
28 days	6.7±1.05	9.9±0.85	6.7±0.00	6.9±3.44

Percentages of syneresis are expressed as mean ± SD. One-way analysis of variance (ANOVA) using Tukey's test was applied to compare mean values of yogurt samples during storage time ($p > 0,05$).

In summary, it can be stated that in the variants of yogurt with the included two strains, separately and in combination, no significant differences in the main physicochemical parameters were determined, and are significantly close to the control variant. This is a good prerequisite for including these two strains with probiotic potential in the composition of starter cultures.

Determination of the changes in viscosity of yogurt samples during storage time

The rheological properties are of particular importance to the quality of the products. The factors that affect the viscosity are the species composition of the strains used in the starter culture, as well as the temperature of incubation. Starter culture is a major factor in changes in yogurt viscosity. A low incubation temperature when obtaining the product at the fermentation stage results in a lower viscosity. A decrease in syneresis leads to an increase in viscosity (Nambiar et al., 2918). When monitoring product quality, viscosity is one of the important parameters, for which it is necessary to examine the product. According to Mok et al., (2008), when the protein gel is formed, the viscosity increases rapidly and then reaches a plateau when the final structure of the fat globules and the aqueous phase is formed. Increasing the structural strength of the protein network is believed to increase the apparent viscosity. Viscosity tracking results of the four tested samples are presented in Figure V.17.

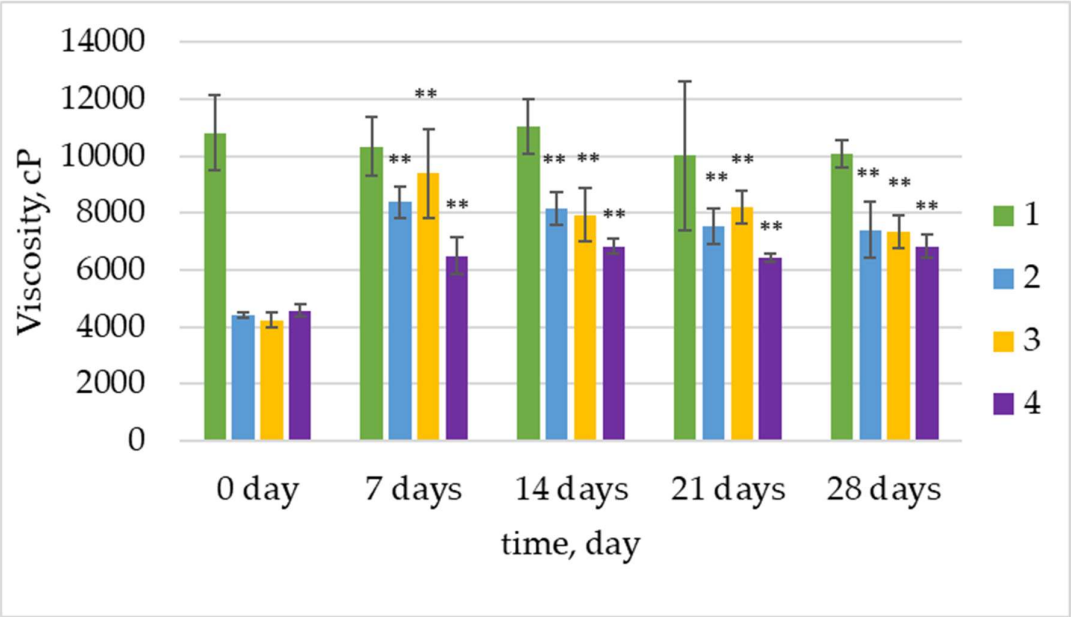


Figure V.17. Viscosity of yogurt during storage time in centipoise (cP)
Data are presented as the mean of replicates ± SD. One-way analysis of variance (ANOVA) using Tukey's test was applied to compare mean values of yogurt samples during storage (** p <0,01).

At day 0 of storage, the yogurt samples showed a lower viscosity compared to the control. The viscosity it was increased significantly after the 7th day of storage, approaching the value of the control variant. Sample 3 inoculated with strain *L. plantarum* KC 5-12 showed a viscosity that was closest to the control sample. Strains of the species *L. plantarum* possess the ability to produce an exopolysaccharide with high thermal stability. The presence of such exopolysaccharides can lead to the improvement of yogurt structure by increasing viscosity (Yang & Yoon, 2022). Yan et al., (2019) indicated that Greek yogurt containing *L. plantarum* had a high viscosity, and the yogurt with the lowest viscosity was the variant with *L. delbrueckii* ssp. *bulgaricus*. In the present study, the lowest viscosity was reported for sample 4, inoculated with a mixture of two strains at a ratio of 1:1 *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3 and *L. plantarum* KC 5-12. These specific differences in results may be due to strain interactions in the combined starter cultures. In the control yogurt sample, no statistically significant difference was found in the determined viscosity during the storage period, while in samples 2, 3, and 4, the viscosity increased on the 7th day of storage with a statistically significant difference, maintaining relative stability up to 28- day of storage.

Viscosity is an important characteristic that determines product texture, but it is also related to water-holding capacity and syneresis (Zayas, 1997). The viscosity of the final product can be significantly affected during the fermentation process. One of the factors that influence the fermentation process, and therefore the viscosity of the product, is the presence of different strains in the used starter culture. The presence of different strains leads to the production of acids with different concentrations and rates, which also determines the dependence of the viscosity of the final product on the starter culture (Oktavia et al., 2016). To determine the relationship between parameters such as water holding capacity, syneresis, pH, TA, and viscosity, Pearson's correlation analysis was performed. The processed results showed that there was a very strong positive correlation between all parameters, with a Pearson correlation coefficient (r) > 0.5.

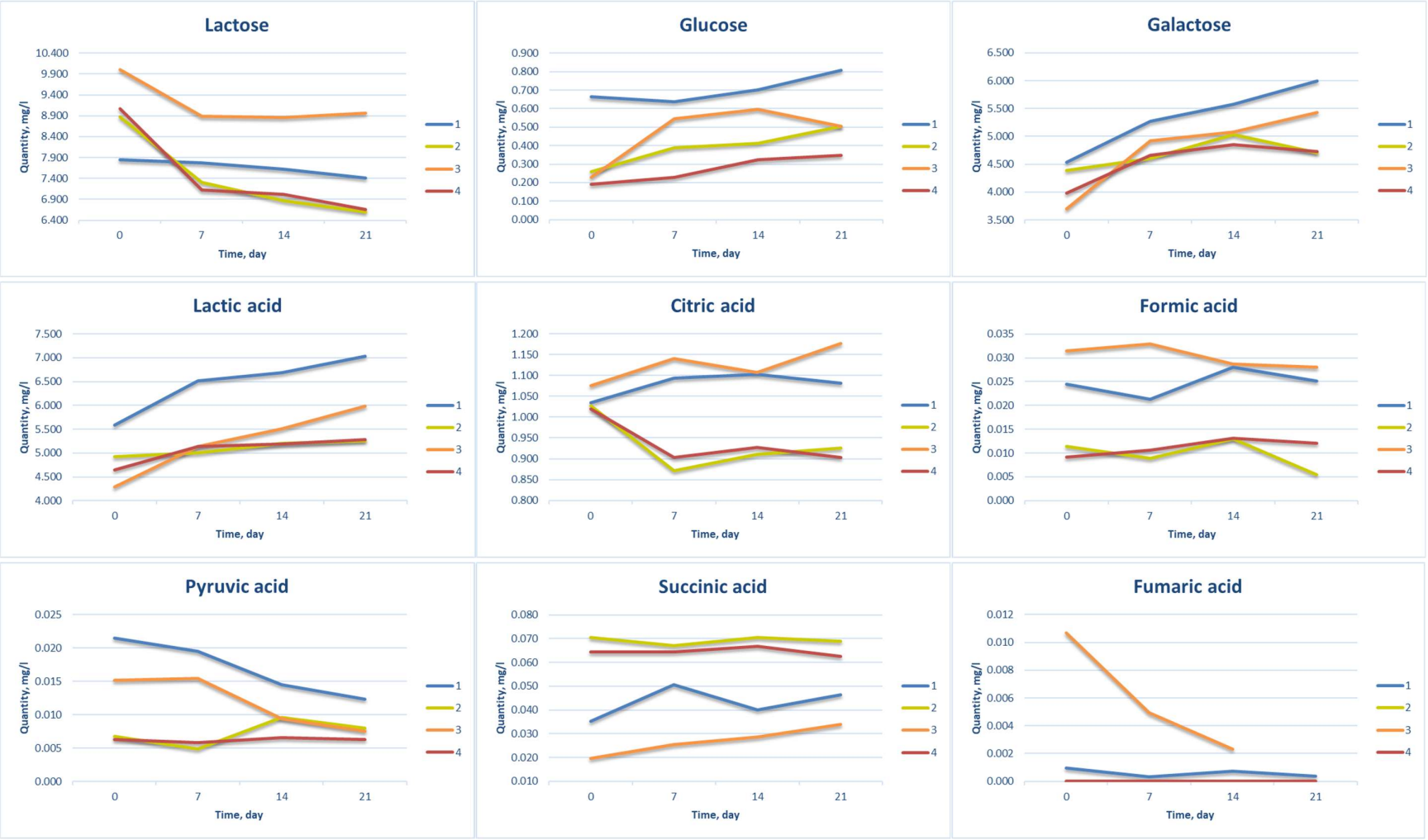
4.3. Investigation of the metabolic profile of the obtained lactic acid products by NMR spectroscopy.

NMR is a technique that is increasingly being used to analyze the chemical components in the food matrix. This technique is also used to analyze yogurt to determine what components are present in the product and to find differences in the metabolic profile when the products are prepared under different conditions, such as the used starter culture, the fermentation conditions, the composition of milk, etc. (Trimigno et al., 2020; Lu et al., 2016).

Supernatants from the prepared four variants of yogurt samples at a total of 4 points during the storage period were analyzed by one-dimensional ^{1}H NMR spectrum and two-dimensional ^{1}H-^{13}C NMR spectrum.

Figure V.18 presents quantitative data of all metabolites detected in the 4 yogurt samples at different time points. In all samples, the presence of the same metabolites was observed, but differences were reported in the amounts and in the dynamics of changes during storage time. A difference was also observed regarding some specific metabolites such as valine, which was absent in sample 3 at all time points, and for the control sample was not reported after the 7th day of storage. The fumaric acid was one component that was reported only in sample 3 and was absent for the other samples. In all variants of yogurt, low molecular weight organic acids were produced. The highest concentration was expected to be lactic acid, while a higher production was reported in the control variant, which can be explained by the symbiont starter culture used. In variant 2, compared to the other variants of yogurt, the highest concentration of acetaldehyde was observed, determining the aromatic characteristics of a product. The amino acids alanine, tyrosine, phenylalanine, and valine were also detected by NMR analysis. The origin of these amino acids in the final products can be related to the starting milk raw material, but due to the observed differences in their concentrations, it is more likely to be the result of the proteolytic activity of the strains themselves used in the preparation of the different yogurt variants.

The presence of such amino acids in products has implications for their functionality and health benefits for consumers. It is important to note that the amino acid phenylalanine was recorded in the variants of yogurt with the two studied strains and it was with the highest concentrations. This amino acid is an important precursor for the synthesis of melanin pigment and neurotransmitters with antidepressant potential (National Research Council, 1989; Lopez & Mohiuddin, 2023; Akram et al., 2020). In the NMR analysis, other components were determined, such as low-molecular organic acids like lactic, citric, formic, and succinic acids, which determine the antibacterial activity of the strains and have implications for the quality of the products (Nes et al., 2012), their functionality and health benefits (Jung et al., 2022). In other similar studies by NMR spectroscopy, similar components were found in yogurt, including lactic acid, citric acid, lactose, galactose, alanine, creatine (Lu et al., 2016), phenylalanine, tyrosine, valine, fumarates and formates (Trimigno et al., 2020). It is also important to note that in the process of storage of the samples, there is an increase, even in very low concentrations, of ethanol, which is also related to preserving the quality of the products.


Lactose
Quantity, mg/l
10.400
9.900
9.400
8.900
8.400
7.900
7.400
6.900
6.400
0
7
14
21
Time, day
1
2
3
4
Glucose
Quantity, mg/l
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
0
7
14
21
Time, day
1
2
3
4
Galactose
Quantity, mg/l
6.500
6.000
5.500
5.000
4.500
4.000
3.500
0
7
14
21
Time, day
1
2
3
4
Lactic acid
Quantity, mg/l
7.500
7.000
6.500
6.000
5.500
5.000
4.500
4.000
0
7
14
21
Time, day
1
2
3
4
Citric acid
Quantity, mg/l
1.200
1.150
1.100
1.050
1.000
0.950
0.900
0.850
0.800
0
7
14
21
Time, day
1
2
3
4
Formic acid
Quantity, mg/l
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
0
7
14
21
Time, day
1
2
3
4
Pyruvic acid
Quantity, mg/l
0.025
0.020
0.015
0.010
0.005
0.000
0
7
14
21
Time, day
1
2
3
4
Succinic acid
Quantity, mg/l
0.080
0.070
0.060
0.050
0.040
0.030
0.020
0.010
0
7
14
21
Time, day
1
2
3
4
Fumaric acid
Quantity, mg/l
0.012
0.010
0.008
0.006
0.004
0.002
0.000
0
7
14
21
Time, day
1
2
3
4

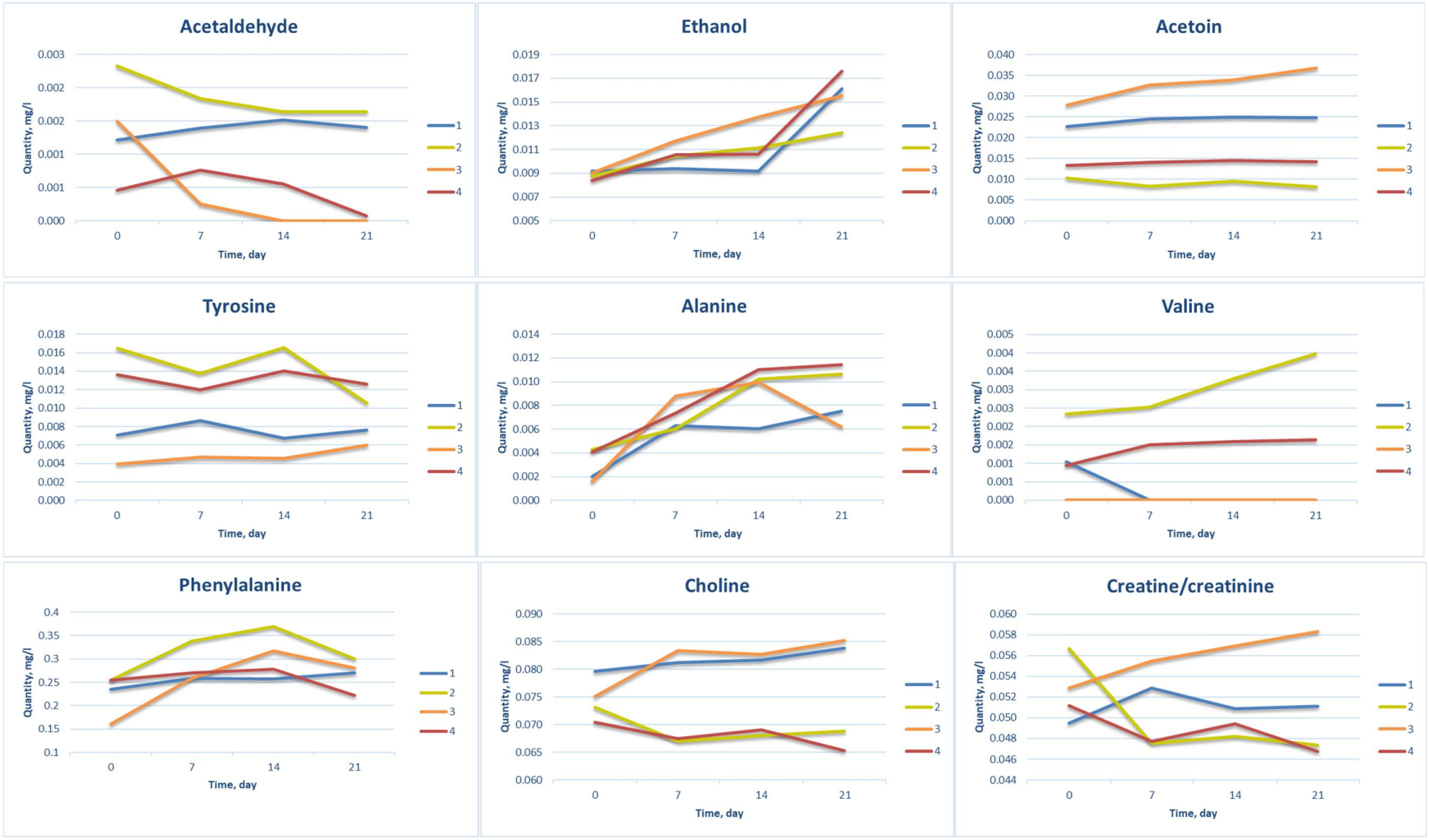


Figure V.18. Quantitative data of all metabolites detected in the 4 yogurt samples at the different time points in NMR analyses

To more clearly determine the differences between the individual variants of the yogurt samples, the data were statistically processed by Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA), which identifies variations associated with a predefined classification, and were represented by a two-dimensional diagram in Figure V.19.

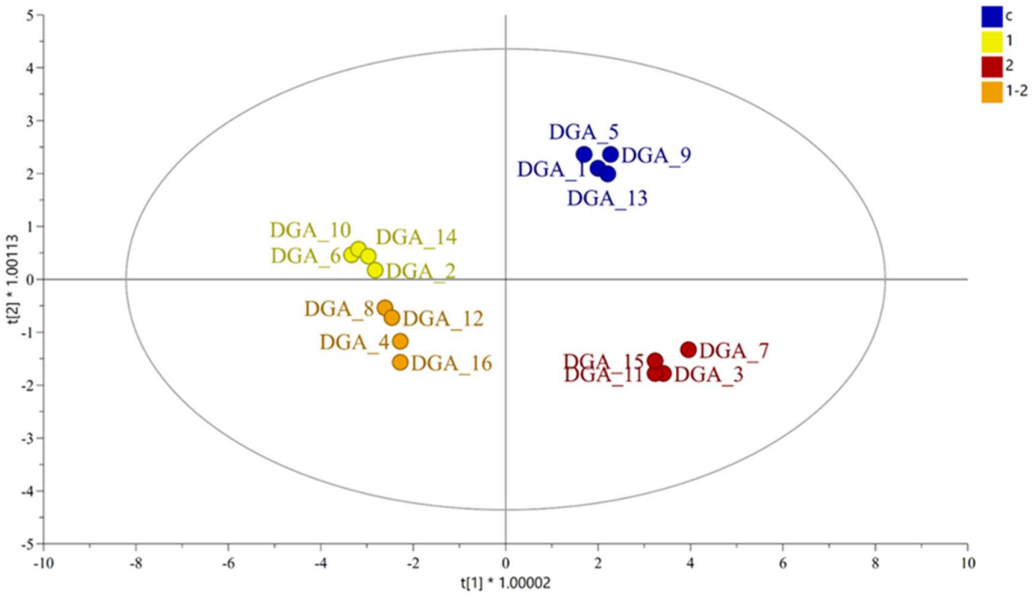


Figure V.19. Two-dimensional plot of the results of the applied OPLS-DA analysis, allowing the differentiation of the yogurt samples at the different storage time points

Legend: in blue – sample 1, control variant with commercial starter culture; in yellow – sample 2; yogurt with an inoculated strain of *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3; in red – sample 3, yogurt with inoculated strain *L. plantarum* KC 5-12; in orange – sample 4, yogurt with inoculated two strains, *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3 and *L. plantarum* KC 5-12 in a ratio of 1:1. DGA_1 - sample 1 at 0 days of storage; DGA_2 - sample 2 at 0 days of storage; DGA_3 - sample 3 at 0 days of storage; DGA_4 - sample 4 at 0 days of storage; DGA_5 - sample 1 on day 7 of storage; DGA_6 - sample 2 on day 7 of storage; DGA_7 - sample 3 on day 7 of storage; DGA_8 - sample 4 on day 7 of storage; DGA_9 - sample 1 on day 14 of storage; DGA_10 - sample 2 on day 14 of storage; DGA_11 - sample 3 on day 14 of storage; DGA_12 - sample 4 on day 14 of storage; DGA_13 - sample 1 at 21 days of storage; DGA_14 - sample 2 at 21 days of storage; DGA_15 - sample 3 at 21 days of storage; DGA_16 - sample 4 at 21 days of storage. Negative values indicate "below average" component scores, even though the values are positive.

The results have shown that the individual variants of the yogurt samples are differentiated, but for each yogurt variant no significant differences were determined at the individual time points, which determines the preservation of the specific characteristics for each of the variants.

Examining different yogurt samples by NMR spectroscopy is a promising method, both for evaluating the potential of inclusion of new strains with functional properties into the starter cultures and for determining and monitoring the quality characteristics of this product. By applying the NMR analysis, the significance of the included new strains in the preparation of the model yogurt products was determined, especially in terms of the production and differences in the concentrations of individual metabolites, which may play a role in determining specific characteristics in each of the variants.

4.4. Viability of LAB in the four variants of yogurt during storage time

LAB present in milk enriches it with nutritional values such as lactic acid, peptides, and amino acids with antimicrobial (Ivanov et al., 2021) and antioxidant activities (Şanlıdere Aloğlu,

H. & Öner, Z, 2011). They break down milk fat to free fatty acids (Nguyen & Hwang, 2016) and produce compounds in the yogurt matrix that contribute to yogurt aroma and flavor (Krastanov et al., 2023; Tian et al., 2020). The most common starter for yogurt production is the combination of two types of bacteria, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (Ivanov et al., 2021; Simova et al., 2008). Strains of the species *L. bulgaricus*, in addition to being used as a starter culture, also possess probiotic properties, showing health benefits to the hosts (Oyeniran et al., 2020). Other species can also be used as starters with probiotic properties, and an example of this is strains of the species *Lactiplantibacillus plantarum*, which enrich yogurt with amino acids, volatile aromatic compounds, and unsaturated fatty acids (Lang, F., et al., 2022; Li, C., et al., 2017). One of the challenges of products with incorporated probiotic strains is to provide a sufficient number of viable cells by the time of consumption (Mani-López et al., 2014).

To monitor the survival of the included strains in the studied variants of the yogurt product, analyses were made to determine the total number of LAB during the storage time at 4°C. The results (Table V.18) have shown the stability of the bacterial culture, as the reported number of viable MO on the selective medium MRS remained 10^8 CFU/g until the end of the storage period. Control sample 1 showed an increase in the number of viable cells on days 7 and 14 of storage, and sample 2 showed an increase on day 7 of storage followed by a decrease on day 28. The number of viable cells in sample 3 also decreased on day 28. Although some statistically significant changes in the number of viable cells were reported for individual samples over the entire storage period, it is important to note that in all variants the strains included as starter cultures remained alive and active (10^8 CFU/g) and can show their functional and probiotic potential up to and including the 28th day. Similar results on the stability of bacteria in yogurt products have been found in many other studies (Fayyaz et al., 2020; Dan et al., 2012; Yan et al., 2019; Mani-López et al., 2014). Shori et al., (2022) reported that in all variants of milk fermented with *Lactobacillus* spp. the highest number of viable cells was found at day 7 of storage and there were no significant changes in the number of viable cells at 21 days. Dimitrellou et al., (2019) also reported that probiotic strains in fermented milk grew well and retained their viability during four-week storage.

Table V.18. Viability of total LAB in four variants of yogurt during storage time

Samples of yogurt	Log10 (CFU/g)				
	0 day	7 days	14 days	21 days	28 days
1	8.55±0.05	8.75±0.02**	8.77±0.04**	8.59±0.07	8.47±0.10
2	8.62±0.09	8.78±0.07*	8.69±0.05	8.52±0.04	8.41±0.06*
3	8.81±0.12	8.84±0.06	8.79±0.05	8.71±0.04	8.50±0.04*
4	8.54±0.05	8.58±0.22	8.61±0.10	8.58±0.03	8.20±0.08

The values of log10 (CFU/g) are the means of triplicate measurements ±SD. One-way analysis of variance (ANOVA) using Tukey's test was applied to compare mean values of yogurt samples during storage (** $p < 0,01$ and * $p < 0,05$).

4.5. Evaluation of the bioprotective potential of LAB strains by co-cultivation with food-associated pathogens

E. coli is a pathogenic bacterium that usually colonizes the intestines of warm-blooded organisms (humans and animals). Primary sources are raw meat products, raw milk, and plant contamination. Some strains of *E. coli* can cause serious illness (WHO, 2018). LAB found in food

products can inhibit the growth of other microorganisms including those that are pathogenic bacteria (Ojo et al., 2017). To evaluate the bioprotective potential, selected strains of LAB were tested by co-cultivation with the pathogen *E. coli* under the conditions of the model products.

Figure V.20 shows the results for evaluating the growth of a test strain of *E. coli* in milk without the presence of LAB as a control and in milk inoculated with a commercial starter culture and the selected strains of *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3 and *L. plantarum* KC 5-12.

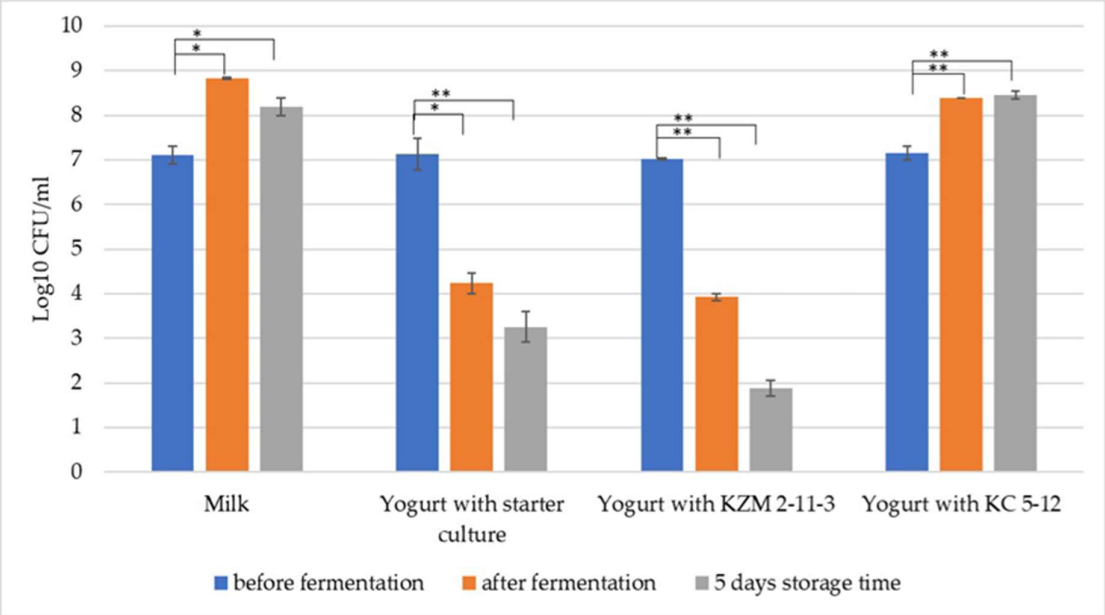


Figure V.20. Inhibition of *E. coli* growth by co-cultivation with selected LAB strains

Results are presented as means ± SD. A one-way analysis of variance (ANOVA) was applied using Tukey's test to compare the mean growth values of *E. coli*, in the samples before, after fermentation and 5 days of storage (** $p < 0.01$ and * $p < 0.05$).

The strain *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3 inhibited the growth of *E. coli*, with almost a 5 log reduction observed in the fermented milk product. After 5 days of product storage, the reduction in *E. coli* was more than 6 log. The sample with strain *L. plantarum* KC 5-12 showed no inhibitory effect on *E. coli* under these experimental conditions. Ojo et al., (2017) reported that LAB strains inhibit diarogenic *E. coli* during co-cultivation with them in yogurt. Fijan et al., (2018), demonstrated that when probiotic strains were co-cultivated with *E. coli*, individual strains had a greater effect in inhibiting the pathogen. The reduction of pH in yogurt is one of the main factors for the antibacterial effect (Fitratullah et al., 2019), and it may depend on the amount of lactic acid and other organic acids in the product, and on other substances with antimicrobial effects such as H_2O_2 (Ito et al., 2003). Ortiz-Rivera et al., (2017) reported that the production of reuterin in a fermented milk product by *L. reuteri* inhibits pathogens and food spoilage microorganisms such as *E. coli* and other pathogens. The studied strain *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3 has positive peroxidase activity when cultured in milk medium (Table V.2), which may be one of the factors for its inhibitory activity against *E. coli* by co-cultivation in yogurt.

In the statistical analysis of the results of the number of viable *E. coli* cells after fermentation and 5 days of storage compared to the inoculated number before fermentation, a

statistically significant difference was found in the strain KZM 2-11-3 and the starter culture samples, confirming a significant effect of inhibiting the growth of *E. coli* (Figure V.20).

4.6. Sensory characteristics of the variants of model yogurt products

Sensory analysis is applied to evaluate product characteristics such as color, surface, presence of liquid above the surface, homogeneity, structure, aroma, and taste. The sensory analysis of the four variants of yogurt samples for the entire storage period of up to 28 days was performed by a group of 15 volunteers who were previously familiar with the sensory characteristics of yogurt according to the National Standard for Bulgarian Yogurt (BDS 12:2010) and the assessment was according to compliance with these characteristics. The results of the obtained estimates are presented in Figure V.21 and Figure V.22. The mean total score of the samples by time points over 7 days of the storage period is presented in Figure V.21, with samples 2 and 4 showing a relatively more stable score with less variation over time and this score is close to to the assessment of the control variant (sample 1). While sample 3 showed some decrease in the total number of points from the 14th day of storage.

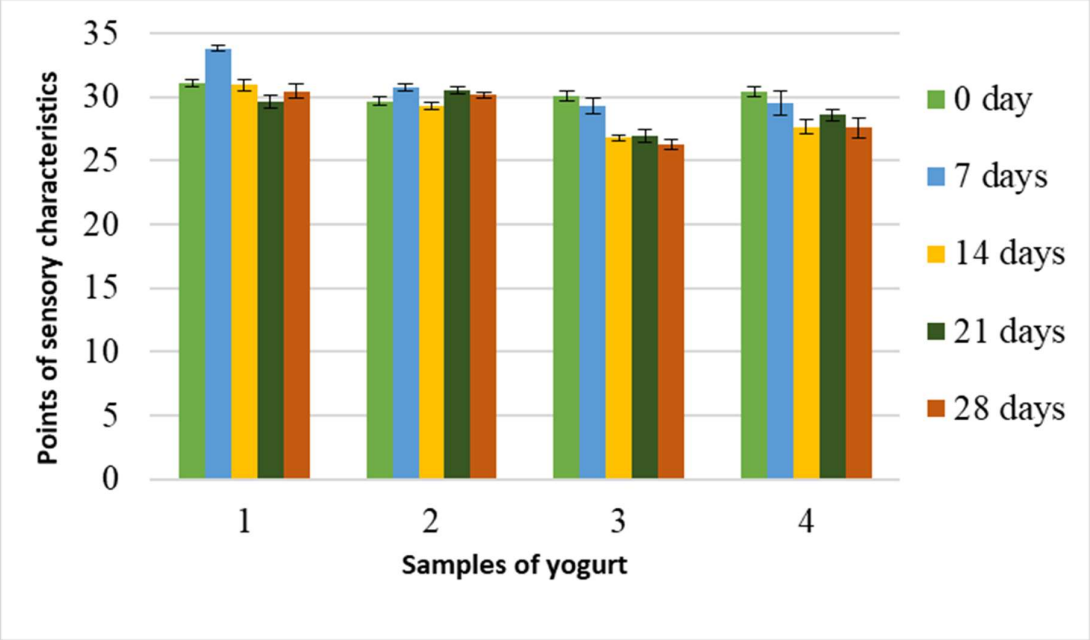


Figure V.21. The mean total scores of yogurt samples during storage ± SD

According to Coggins et al. (2008), taste and texture are some of the most important characteristics that determine differences in yogurt preferences. Aroma, sweetness, acidity, chalky mouthfeel, and viscosity have also been identified as significant characteristics in fermented milk products (Allgeyer et al., 2010).

By each sensory characteristic of the products evaluated during storage, i.e. 0 day, 7 days, 14 days, 21 days, and 28 days, the average value was calculated and the results are presented in Figure V.22. In sample 2 with inoculated strain *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3, the most uniform distribution was observed according to the scores obtained for the individual characteristics, and they were the closest to those of the control yogurt variant.

The panel of assessors identified sample 2 as the variant with the best color, aroma, and taste of the three experimental samples, and this result was also consistent with the metabolic profile of this variant including aroma-determining components (Figure V.18). Sample 3,

inoculated with strain *L. plantarum* KC 5-12, was rated for the best homogeneity. Sample 4, with both inoculated strains, was generally rated with intermediate sensory characteristics to those of samples 2 and 3, with the highest ratings for smooth and shiny surface and texture.

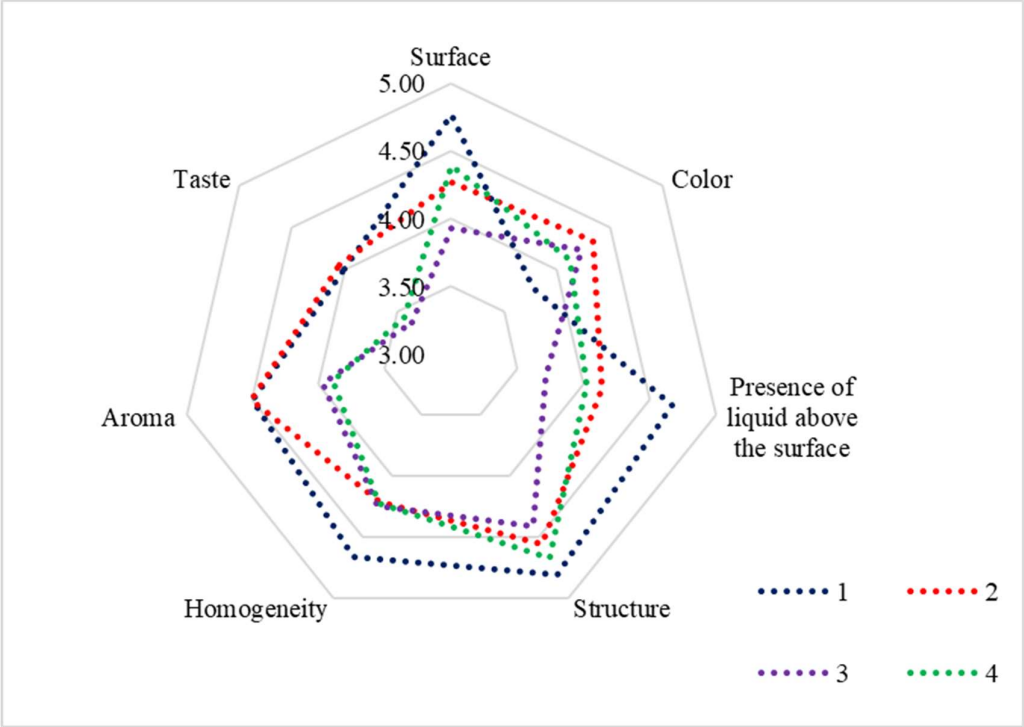


Figure V.22. Evaluation of sensory indicators of yogurt samples

The selection of two strains of *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3 and *L. plantarum* KC 5-12 for inclusion in a model yogurt product was made based on their characteristics showing that the two strains have probiotic potential and bioprotective properties. When analyzing a series of sensory indicators in the prepared variants of yogurt products, the samples with the studied strains did not differ in most physicochemical parameters from the control sample, especially at the end of the fermentation process and during the storage period. It is important to emphasize that in strain *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3 a highly pronounced effect was observed in inhibiting the growth of *E. coli* in the product, and this strain also showed inhibitory activity against food contaminants such as the yeasts *K. marxianus, K. lactis* and *S. cerevisiae*, whose presence in products can contribute to significant changes in quality and sensory characteristics. In strain *L. plantarum* KC 5-12, the highest probiotic potential of the entire group of strains was determined and, in parallel, it exhibited a well-defined effect on the inhibition of the growth of yeast and mold contaminants (more than 60%).

In conclusion, it can be summarized that the use of *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3 and *L. plantarum* KC 5-12 as strains with bioprotective and probiotic potential included in the composition of production starter cultures is very promising. With enriched starter cultures, new food products can be produced with enhanced functionality and health benefits for consumers and with preserved quality for the entire storage time.

CONCLUSIONS

1. Twelve new strains were isolated from the natural microbiota of traditionally prepared fermented foods, of which 7 strains are of the species *Lactiplantibacillus plantarum*, 2 strains are of the species *Lactobacillus delbrueckii* ssp. *bulgaricus*, 1 strain is *Loigolactobacillus coryniformis*, 1 strain is identified as *Latilactobacillus sakei* and 1 strain is *Pediococcus pentosaceus*.

2. The newly isolated strains are characterized by certain antimicrobial activity against Gram (+) and Gram (-) test-pathogenic bacteria and a pronounced inhibitory effect against yeast and mold food-associated contaminants. Five of the newly isolated strains exhibited antiviral activity against HHV, as for two strains *Lactobacillus delbrueckii* ssp. *bulgaricus* KZM 2-11-3 and *Lactiplantibacillus plantarum* KC 5-12 a selective index (SI) above 45 was determined against the HHV-2 virus model.

3. The newly isolated strains were characterized by a well-expressed aminopeptidase enzyme profile, but genetic determinants for the studied peptidases were found only at strain *Latilactobacillus sakei* C 10-31-3, which supports the hypothesis for species-specificity in designing of primers for peptidase genes in LAB.

4. Multiantibiotic resistance was observed in the studied strains, but the presence of tet(M), erm(B), and cat genes, which are indicated as the most controlled for the assessment of the potential for transfer of antibiotic resistance according to the Qualified Presumption of Safety (QPS) of EFSA, was not detected.

5. Pronounced auto- and co-aggregation abilities are characterized by the strains *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-1, *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3, *L. plantarum* KC 5-12, and *L. sakei* C 10-31-1. The best adhesive abilities have shown strains *L. coryniformis* KO 3-7-5, *P. pentosaceus* KC 5-13, *L. plantarum* KC 5-14, and *L. plantarum* KZC 8-21-1. It is important to emphasize that the two *L. delbrueckii* ssp. *bulgaricus* strains exhibit adhesive properties too, such as not usually described for other strains of this species.

6. In most of the studied strains, a survival level of over 50% is reported under the direct impact of the stress factors, typical for the upper departments of the GIT. All strains retain the ability to grow in the presence of pancreatin and have a good ability to survive under direct impact of bile salts, as at three strains *L. plantarum* KC 5-12, *P. pentosaceus* KC 5-13, and *L. plantarum* KZC 8-23 -5 the inhibition factor is less than 0.4.

7. The best expressed probiotic potential was determined at the strain *L. plantarum* KC 5-12, followed by strains *L. plantarum* KC 5-14, *L. plantarum* KZC 8-21-1, and *P. pentosaceus* KC 5-13. A significant probiotic potential was also determined for the strain *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3.

8. The studied strains show a very good level of survival at the technological process of freeze-drying and subsequent storage with an appropriately selected protective media.

9. No significant differences with the control variant were found in the yogurt model products with the two selected strains, *Lactobacillus delbrueckii* ssp. *bulgaricus* KZM 2-11-3 and *Lactiplantibacillus plantarum* KC 5-12, in regard to the main physicochemical parameters,

while the viscosity of the samples increased on the 7th day of storage with a statistically significant difference and remained relatively stable until the 28th day of storage.

10. The strains included in the model yogurt products show differences in the production and concentrations of individual metabolites that contribute to the specific characteristics of each variant. According to the metabolic profile, the variants are differentiated from each other, and for each of them, no significant differences were found for the storage period.

11. The strains applied as starter cultures in the yogurt model products are kept alive and active and can show their functional and probiotic potential until the end of the storage period.

12. Strain *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3 exhibited a pronounced bioprotective effect by inhibiting the growth of *E. coli* by almost 5 log in the fermented milk product and by more than 6 log after 5 days of product storage.

13. At the model product, yogurt with an inoculated strain of *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3 was observed to have the most uniform distribution of the evaluations of individual sensory characteristics, which were the closest to those of the control variant, and it was rated with the best color, aroma, and taste, which corresponds to the aroma-determining components from the metabolic profile.

CONTRIBUTIONS

1. The collection of 12 newly isolated strains of lactic acid bacteria was collected from samples of traditionally prepared fermented foods, for which species affiliation and basic physiological, functional, and technological characteristics were determined.

2. A complex approach was applied to evaluate the probiotic potential and bioprotective characteristics of the newly isolated strains.

3. Antiviral activity against the human herpes virus was found for the first time in a strain of *L. delbrueckii* ssp. *bulgaricus*.

4. A model yogurt product with two selected and successfully applied strains was developed, and their significance and role in forming a specific metabolic profile and sensory characteristics were confirmed.

5. The applicability of NMR spectroscopy has been confirmed as a high-tech method for clearly defining different types of yogurt according to their specific metabolic profiles, which is due to the strains in the composition of the starter cultures.

6. The applicability of the strains *L. delbrueckii* ssp. *bulgaricus* KZM 2-11-3 and *L. plantarum* KC 5-12 with bioprotective and probiotic potential have been proven for inclusion in the starter cultures for the production of new functional food products with health benefits for consumers and preserved quality throughout the storage period.

PUBLICATIONS

1. Ramize Hoxha, Yana Evstatieva, Dilyana Nikolova, New lactic acid bacterial strains from traditional fermented foods - bioprotective and probiotic potential, Journal of Chemical Technology and Metallurgy, vol:58, issue:2, 2023, pages:252-269, ISSN (print):1314-7471, ISSN (online):1314-7471, SCOPUS, SJR (0.196 - 2022), SCOPUS Quartile: Q3 (2022),

Cited in:

Perveen, I., Bukhari, B., Sarwar, A., Aziz, T., Koser, N., Younis, H., Ahmad, Q.U.A., Sabahat, S., Tzora, A. and Skoufos, I., 2023. Applications and efficacy of traditional to emerging trends in lacto-fermentation and submerged cultivation of edible mushrooms. Biomass Conversion and Biorefinery, pp.1-20. https://doi.org/10.1007/s13399-023-04694-9 , in WoS / SCOPUS

Nasr, N.M., Abd-Alhalim, L.R. Characterization and identification of *Lactobacillus rhamnosus* and *Enterococcus durans* as probiotic potential isolated from selected dairy products in Egypt. J. Umm Al-Qura Univ. Appll. Sci. (2023). https://doi.org/10.1007/s43994-023-00090-1 , in WoS / SCOPUS

2. Ramize Hoxha, Daniel Todorov, Anton Hinkov, Kalina Shishkova, Yana Evstatieva, Dilyana Nikolova, In Vitro Screening of Antiviral Activity of Lactic Acid Bacteria Isolated from Traditional Fermented Foods, Microbiology Research, vol:14, issue:1, 2023, pages:333-342, ISSN (online):2036-7481, https://doi.org/10.3390/microbiolres14010026 , IF (1.5 - 2022)

Cited in:

Khan, A.; Li, X.; Weidong, W. Probiotic Fermented Food Inhibits Viral Infection; a Concept of Modern Era. Preprints 2024, 2024010203. https://doi.org/10.20944/preprints202401.0203.v1

3. Ramize Hoxha, Yana Evstatieva, Dilyana Nikolova, Physicochemical, Rheological, and Sensory Characteristics of Yogurt Fermented by Lactic Acid Bacteria with Probiotic Potential and Bioprotective Properties, Foods, vol:12, issue:13, 2023, pages:2552-0, ISSN (print):2304-8158, https://doi.org/10.3390/foods12132552 , IF (5.2 - 2022), Web of Science Quartile: Q1 (2022), SCOPUS, SJR (0.771 - 2022), SCOPUS Quartile: Q1 (2022)

Cited in:

Gomaa, M.A., Allam, M.G., Mokhtar, E., Ayad, E.H., Darwish, S.M. and Darwish, A.M., 2023. Nano casein–pectin complex: exploring physicochemical, organoleptic properties, and LAB viability in skimmed milk and low-fat yoghurt. Frontiers in Nutrition, 10. https://doi.org/10.3389/fnut.2023.1288202 , in WoS / SCOPUS

Younas, S., Murtaza, M.A., Manzoor, M.S., Arqam, U., Ali, Z., Hafiz, I., Anees Ur Rehman, M. and Imran, M., 2024. Effect of probiotic incorporation on physicochemical attributes of yogurt during storage and influence on cholesterol assimilation. Journal of Food Science. ISSN 00221147 https://doi.org/10.1111/1750-3841.16898, in WoS / SCOPUS

Mittal, A., Benjakul, S., Palamae, S., Saetang, J. and Singh, A., Milk fortified with chitooligosaccharide-gallic acid conjugate and its liposome: Characterisation, storage stability and in vitro digestibility. International Journal of Food Science & Technology. https://doi.org/10.1111/ijfs.16895 , in WoS / SCOPUS

4. Ramize Hoxha, Dilyana Nikolova, Yana Evstatieva, Antibiotic resistance profile of the newly isolated lactic acid bacteria strains from traditional fermented foods, Current Trends in Natural Sciences, vol:11, issue:21, 2022, pages:247-253, ISSN (print):2284-9521, ISSN (online):2284-953X, https://doi.org/10.47068/ctns.2022.v11i21.027

Cited in:

ENCU, Ş. B., ACAR SOYKUT, E., & ÇAKIR, İ. (2022). Geleneksel yoğurtlardan izole edilen laktik asit bakterilerinin MALDI TOF MS Biotyper sistemi ile tanimlanmasi ve bazi starter kültür özelliklerinin belirlenmesi. Gıda, 47(6), 1059-1082. https://doi.org/10.15237/gida.GD22088 .