SÜLE Judit[1], VARGA László[2,3], HATVAN Zoltán[2], KERÉNYI Zoltán[1]

DOI: https://doi.org./10.52091/EVIK-2023/1-2-ENG

Received: June 2022 - Accepted: November 2022

In vitro *evaluation of certain probiotic properties of lactic acid bacteria isolates*

Keywords: probiotic, lactic acid bacteria, antibiotic resistance, antimicrobial activity

1. Abstract

Bacteria carrying genes responsible for antibiotic resistance cannot be used in food production. For this reason, exploring the antibiotic resistance profile of probiotic candidates and the antimicrobial substances they produce are essential for probiotic strain selection. The aim of this study was to develop and evaluate additional elements of a complex *in vitro* test system for rapid and efficient selection of a large number of putative probiotic isolates. In a previous work, we had tested bacterial strains (n=217) isolated from Transylvanian raw sheep milk, cultured sheep milk, and sheep cheese samples and we reduced the sample number to a total of six Gram-positive, non-hemolytic, catalase-negative, well-aggregating, good acid and bile acid tolerating strains. In this research, we investigated the antibiotic resistance and antimicrobial production capacity of the pre-selected strains (n=6). The antimicrobial activity of the isolates was determined by the agar well diffusion assay. Strains E15, E66, E173, E198, and E216 were found to inhibit the growth of both *Salmonella Enteridis* ATCC 13076 and the control strain (i.e., *Lactobacillus acidophilus* ATCC 4356). Antibiotic resistance tests were performed by the agar disk diffusion method. All six isolates belonging to the species of *Levilactobacillus brevis* and *Lactiplantibacillus plantarum* were found to be resistant to several antibiotics and, therefore, cannot be used for the manufacture of commercial probiotic products. In conclusion, our in vitro test system proved to be capable of effectively screening out unsafe isolates.

[1] Hungarian Dairy Research Institute Ltd.
[2] Széchenyi István University, Albert Casimir Faculty at Mosonmagyaróvár, Department of Food Science
[3] Széchenyi István University, Wittmann Antal Multidisciplinary Doctoral School in Plant, Animal, and Food Sciences

Judit SÜLE	jsule@mtki.hu	https://orcid.org/0000-0002-7202-9748
László VARGA	vargal@mtk.nyme.hu	https://orcid.org/0000-0001-7431-852X
Zoltán HATVAN	info@ovarigazdasz.hu	https://orcid.org/0000-0002-3885-4904
Zoltán KERÉNYI	kerenyiz68@gmail.com	https://orcid.org/0000-0001-8665-5216

2. Introduction

Probiotics are viable microbes - typically, but not exclusively, bacteria - that, when administered in sufficiently large quantities, exert positive effects on human or animal health **[1, 2, 3, 4]**. Some of their physiological benefits (e.g., restoration of damaged microbiota, competitive exclusion of pathogens, production of acids and short-chain fatty acids) are widespread in the well-known and extensively studied probiotic genera, whereas others (e.g., neutralization of carcinogens, strengthening of the intestinal barrier) are common in the majority of strains of a probiotic species (so-called species-level effects), while a third group of beneficial effects (e.g., neurological, immunological, and endocrinological effects) are rare and occur in only a few strains of a given species (so-called strain-level effects) **[2].**

A large number of bacterial strains are being isolated worldwide with the aim of finding strains with superior properties that are believed to be probiotic. Expensive and complicated animal studies must be preceded by a pre-selection system of *in vitro* studies **[5]**, which can quickly, easily, and relatively cheaply select strains - from thousands of isolates - that are hoped to be probiotic in subsequent *in vivo* experiments **[6, 7, 8].**

In a previous study, we tested bacterial strains (n=217) isolated from raw milk, cultured milk and cheese samples produced in Transylvania and found a total of 6 Gram-positive, non-hemolytic, catalase-negative, well-aggregating, good acid and bile acid tolerating strains **[9]**. We also aimed to test one isolate (E10), which is not well-aggregating and has poor acid and bile acid tolerance. In this work, we investigated the antibiotic resistance and antimicrobial production capacity of the pre-selected strains (n=6+1). Bacteria carrying genes responsible for antibiotic resistance cannot be used for food production, therefore, the elucidation of the antibiotic resistance profile of probiotic strains and the knowledge of the antimicrobial substances they produce is essential for the selection of probiotic strains **[10].**

With this in mind, the aim of the present research was to develop and evaluate further elements of the *in vitro* test system, which included the assessment of antibiotic resistance and antimicrobial activity of the selected bacterial strains and their genotyping. Ultimately, we aimed to find probiotic strains that show antimicrobial activity against pathogenic microorganisms, while not being resistant to any antibiotics and genetically belonging to the genus *Lactobacillus* or one of its descendant genera **[11].**

3. Materials and methods

3.1 Bacterial strains used in this study

As mentioned above, based on the results of a previous work **[9]**, seven bacterial strains isolated from raw sheep milk, fermented sheep milk and sheep cheese samples produced in Transylvania were included in this study. The selected strains represented a single clonal group as determined by RAPD-PCR. Six of the isolates performed excellently in the classical microbiological tests (i.e., colony morphology, Gram staining, catalase assay, hemolysis test), the auto-aggregation test and the acid and bile acid tolerance tests, whereas the seventh (E10) was included in our further experiments because it performed the least well in all the tests based on preliminary results. Our intention was to find out if it would perform better in further tests or if it would continue to significantly underperform the other strains in all important traits.

Isolates were preserved and stored in glycerol stock solution. A loopful of the strain taken from the surface of MRS-CC agar or MRS pH 5.4 agar was washed into 3 ml of medium and incubated according to the requirements of the specific strain. 300 µl of bacterial culture and 900 µl of 60% glycerol solution were added to a freezer tube. They were vortexed and then frozen in liquid nitrogen for about 30 s. Storage was at -80 °C in an ultra-low freezer. Strains were revived and cultured as shown in ***Table 1.***

Table 1. Revival and maintenance conditions of isolated and control bacterial strains involved in this study

Strain	**Internal identifier**	**M / T / P / C***
Lacticaseibacillus paracasei subsp*. tolerans* NBRC 15906**	E10	MRS / 37 ± 1 / 72 / AN
Levilactobacillus brevis ATCC 14869**	E15	MRS / 37 ± 1 / 72 / AN
Levilactobacillus brevis ATCC 14869**	E66	MRS / 37 ± 1 / 72 / AN
Levilactobacillus brevis ATCC 14869**	E92	MRS / 37 ± 1 / 72 / AN
Levilactobacillus brevis ATCC 14869**	E173	MRS / 37 ± 1 / 72 / AN
Levilactobacillus brevis ATCC 14869**	E198	MRS / 37 ± 1 / 72 / AN
Lactiplantibacillus plantarum JCM 1149**	E216	MRS / 37 ± 1 / 72 / AN

Strain	Internal identifier	M / T / P / C*
Lactobacillus acidophilus ATCC 4356	B1	MRS / 37 ± 1 / 72 / AN
Lactococcus lactis subsp. *lactis* ATCC 11454	B4	MRS / 37 ± 1 / 72 / AN
Lactobacillus acidophilus LA-5	B10	MRS / 37 ± 1 / 72 / AN
Escherichia coli ATCC 8739	B40	CASO / 37 ± 1 / 24 / A
Staphylococcus aureus subsp. *aureus* ATCC 49775	B41	CASO / 37 ± 1 / 24 / A
Salmonella enterica subsp. *enterica* serovar. Enteritidis ATCC 13076	B51	CASO / 37 ± 1 / 24 / A

*M: maintenance medium, T: incubation temperature (°C), P: incubation period (hour), C: incubation conditions (A: aerobic, AN: anaerobic).

**Bacterial isolates were identified to species level, based on the results of genetic tests, at the end of the study.

3.2. Conditions and media used for selective culturing

3.2.1. Physiological saline solution

For the dilution fluid used to prepare the decimal dilution series, 8.5 g of NaCl was added and dissolved in 1 L of distilled water. Sterilization was carried out in an autoclave at 121 °C for 15 min. The casein peptone solution was prepared in a similar manner with the addition of 1 g of tryptone (casein peptone).

3.2.2. Phosphate buffer solution (PBS)

We used commercially available 1 × PBS solution (Biolab Zrt., Budapest, Hungary), which was sterilized at 121 °C for 15 min before use.

3.2.3. De Man-Rogosa-Sharpe (MRS) agar and broth (pH 6.2)

The commercially available MRS agar and broth (Oxoid, Basingstoke, UK) were prepared according to the manufacturer's instructions. The recommended amounts (62 g and 52 g, respectively) were measured analytically and dissolved in 1 L of distilled water each. A heated magnetic stirrer was used to assist dissolution of the components, followed by sterilization of the media in an autoclave (121 °C for 15 min). The pH (6.2) was checked after sterilization.

3.2.4. M17 agar and broth (according to Terzaghi)

Following the manufacturer's instructions (Biokar Diagnostics, Allonnen, France), 57.2 g of dehydrated M17 agar and 42.2 g of broth were weighed on an analytical balance and dissolved in 1000 ml of distilled water each. The culture media were heated until the components were completely dissolved, then transferred to heat-resistant vials and sterilized in an autoclave at 121 °**C** for 20 min.

3.2.5. CASO agar and broth

CASO agar and CASO broth were also prepared according to the manufacturer's (Biolab) instructions. 45 g and 36 g, respectively, were dissolved in 1 L of water each. After dissolution, sterilization was performed in an autoclave under standard parameters (121 °C, 15 min).

3.2.6 Anaerobic culturing

Anaerobic conditions were generated during the trials as follows: agar plates were incubated in AnaeroPack Rectangular jars (Merck, Darmstadt, Germany) with GENbox anaerobic salt (bioMériux, Marcy-l'Étoile, France). The presence of anaerobic conditions was indicated by the change from white to blue color of the Microbiologic Aerotest indicator (Merck).

3.3. Antimicrobial activity testing

3.3.1. Materials and tools needed for this study

3.3.1.1. CASO agar

It was prepared as described in subsection 3.2.5.

3.3.1.2 Afilact® Fluid lysozyme enzyme

The active ingredient in the preservative, produced by Chr. Hansen (Hørsholm, Denmark), is lysozyme, which inhibits the growth of a number of Gram-positive bacteria. Lysozyme is an approved food

ingredient in more than 30 countries. The product contains an enzyme extracted from egg white (E1105) and is suitable for use in the EU for the preservation of cheese.

3.3.1.3. Flóraszept

Flóraszept (Unilever, Budapest, Hungary) is a disinfectant. Its main active ingredient is sodium hypochlorite, which is effective against both bacteria and fungi. The product was used undiluted in our experiments.

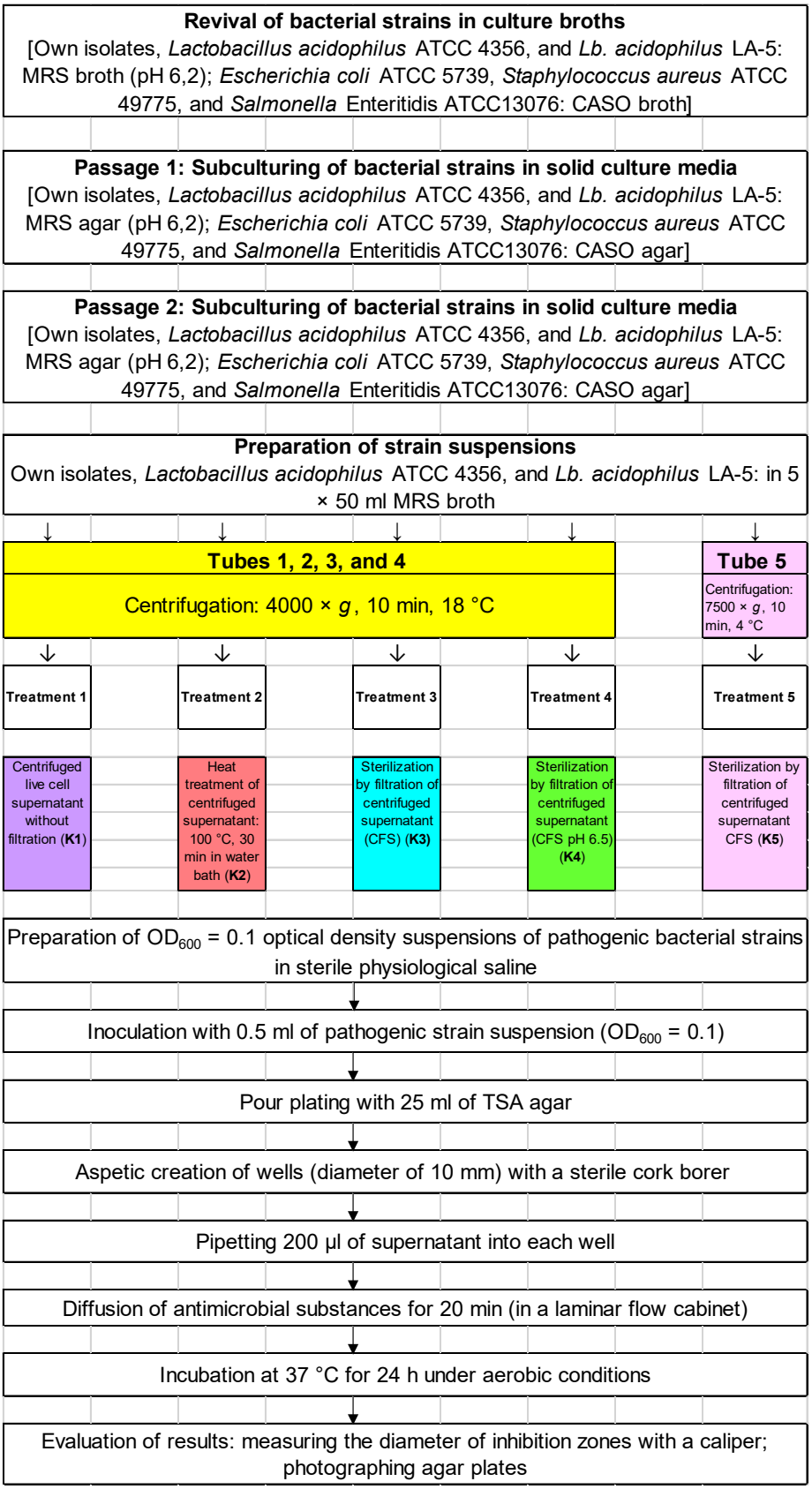

Figure 1. The process of antimicrobial activity testing

3.3.2. Agar well diffusion assay

3.3.2.1. Preparation of pathogenic bacteria suspensions

The bacterial strains were revived and then passed twice. After the second passaging, 24 h fresh cultures were used to prepare suspensions. 10 ml of sterile distilled water was pipetted onto the colonies grown on the medium and the colonies were carefully loosened from the agar surface using a flat-ended cell spreader. The suspensions were then pipetted from the Petri dishes into plastic Falcon tubes. Cell densities were adjusted, based on the principle of optical density, by measuring at 600 nm wavelength with a BioMate 160 UV-Vis spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA). The values were standardized to 0.1 for each sample to allow comparison of the measurement results.

3.3.2.2. Preparation of agar plates

0.5-0.5 ml of the bacterial suspensions prepared as described above were pipetted into sterile Petri dishes and plates were then poured with CASO agar cooled to 45 °C. To ensure uniform distribution of microbes in the medium, gentle circular rotating movements were performed upon pouring of agar plates. After the culture medium had solidified, 4 wells of 10 mm in diameter were punched in each agar plate using a sterilized cork borer. The agar disks were aseptically removed from the plates.

3.3.2.3. Treatment of own isolate supernatants

200 µl of sterile physiological saline was pipetted into the first of the four wells. This served as a control because it did not inhibit bacterial growth, indicating that the strain was in a viable state. In the other three wells, 200 µl aliquots of the supernatant from one of the treatments of a specific isolate was added, thus achieving three technical replicates on one plate. The plates were then incubated according to the requirements of the particular bacterial pathogen.

The supernatants of the isolates to be tested were subjected to five different treatments. The first phase of the treatments was identical in that five Falcon tubes each were filled with 45 ml of MRS broth (De Man, Rogosa, Sharpe broth) and each strain was thus revived under optimum growth conditions (i.e., 37 °C, 24 h, anaerobiosis). After incubation, however, the sample preparation procedures were different as shown in ***Figure 1.***

Treatment 2: The steps were the same as in treatment 1, but after pouring the contents of the second set of Falcon tubes into a sterile Falcon tube, these supernatants were heat treated (in a water bath set at 100 °C for 30 min).

Treatment 3: The steps were the same as in treatment 1, but for samples from Falcon tubes no. 3, after the second centrifugation, the supernatants were poured into a plastic urine collection cup and filtered through a membrane filter with 0.2 µm pore size into a sterile 50 ml Falcon tube.

Treatment 4: After a single centrifugation (4000 *g*, 10 min, 18 °C) of Falcon tubes no. 4, the culture medium was poured into new sterile Falcon tubes. The pH of the supernatant (containing the broth) was then adjusted to 6.5. 1 M NaOH was used to reach the desired value, and the pH was checked with a FiveEasy F20 pH meter (Mettler Toledo, Columbus, OH, USA). After adjustment, the supernatant was filtered through a membrane filter with 0.2 µm pore size into a sterile 50 ml Falcon tube.

Treatment 5: The centrifugation parameters of Falcon tubes no. 5 were different from those used in the previous treatments. The samples were centrifuged at 7500 *g for* 10 min at 4 °C, and the culture medium was also drained and filled with 42 ml of 1 × PBS solution. Then centrifugation followed according to the modified settings. The supernatant was poured into a plastic urine collection beaker and filtered through a membrane filter with 0.2 µm pore size into a sterile 50 ml Falcon tube.

3.3.2.4 Incubation and measurement of inhibition zones

Following 20 min of diffusion, the plates were placed in a thermostat and incubated according to the requirements of the pathogenic bacteria. The diameter of inhibition zones was measured with a caliper. Each test was performed with two parallel plates. Two types of outcomes were observed, i.e., either an inhibition zone developed around the well containing the supernatant or no change was visible around it. The latter meant that the bacterial supernatant neither inhibited nor stimulated bacterial growth.

3.4. Antibiotic susceptibility testing

3.4.1. Materials and tools needed for the study

3.4.1.1 De Man-Rogosa-Sharpe (MRS) agar (pH 6.2)

It was prepared as described in subsection 3.2.3.

3.4.1.2. Iso-Sensitest agar

As recommended by the manufacturer (Oxoid), 31.4 g of product was dissolved in 1 L of water. After complete dissolution of the components, the media were sterilized at 121 °C for 15 min.

3.4.1.3 *Lactobacillus* Susceptibility Medium (LSM)

MRS (Oxoid) and Iso-Sensitest (Oxoid) media were required for the preparation of LSM agar. 900 ml of Iso-Sensitest broth and 100 ml of MRS broth were mixed together and 15 g of agar-agar was also added. Sterilization was carried out under standard parameters (121 °C, 15 min).

3.4.1.1.4. Antibiotic-impregnated filter paper disks

The characteristics of the antibiotic susceptibility disks (Biolab) used in this study are summarized in ***Table 2.***

Table 2. Active substance concentrations of antibiotic sensitivity disks used for antibiotic resistance testing

Name of antibiotic	Concentration of active substance (µg)	Abbreviation
Ampicillin	10	AM10
Chloramphenicol	30	C30
Clindamycin	2	DA2
Erythromycin	15	E15
Gentamicin	10	CN10
Kanamycin	30	K30
Nalidixic acid	30	NA30
Streptomycin	10	S10
Tetracycline	30	TE30
Trimethoprim + Sulfamethoxazole	1.25 + 23.75	SXT25
Vancomycin	30	VA30

3.4.2 Process for antibiotic resistance testing

Figure 2 illustrates the steps of the antibiotic resistance tests performed.

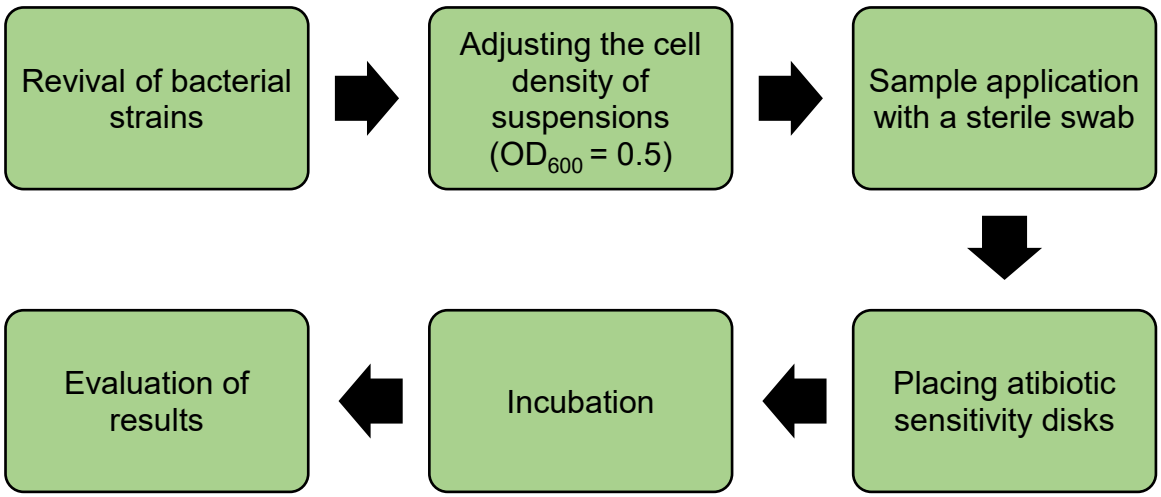

Figure 2. Scheme of antibiotic resistance testing

We revived our bacterial strains on MRS medium and incubated them under optimal conditions. We then inoculated a solitary colony onto the surface of a new MRS medium to ensure that we were starting from a single colony and not working with mixed cultures. After 24 h, 10 ml of distilled water was pipetted onto the grown colonies to dissolve them, taking care not to mix medium into the suspension. The solution was pipetted from the top of the Petri dish into a Falcon tube. The density of the cell suspensions was then adjusted to OD_{600} = 0.5 using a BioMate 160 UV-Vis spectrophotometer. For each dilution, we checked

whether our calculations were correct. Cell suspensions were then applied to the surface of the media (MRS agar, ISO-Sensitest agar, LSM agar) with sterile swabs in such a way that the entire surface of the media was covered by the applied sample volume. The Petri dishes were allowed to stand for 15 min to let the microbes adapt to the conditions. Six antibiotic resistance disks (including 1 blank disk) were placed in each Petri dish, which were then incubated under optimum conditions (37 °C, 24 h, anaerobiosis). After 1 d of incubation, the inhibition zones were measured with a caliper. The diameter of the disk (6 mm) was always subtracted from the size of the zones.

3.5. Genetic identification

3.5.1. Materials

The specific materials used for genetic identification included:

- Bacterial cultures on the surface of agar media
- NucleoSpin Microbial DNA isolation kit (Macherey-Nagel kit)
- DreamTaq Green PCR Master Mix 2×
- 27f and 1492r primers
- Agarose gel
- EcoSafe nucleic acid staining solution
- Gene Ruler 1kb plus DNA ladder
- GeneJet PCR purification kit (Thermo Fisher)

3.5.2. Identification process

The steps of genetic identification are shown in ***Figure 3.***

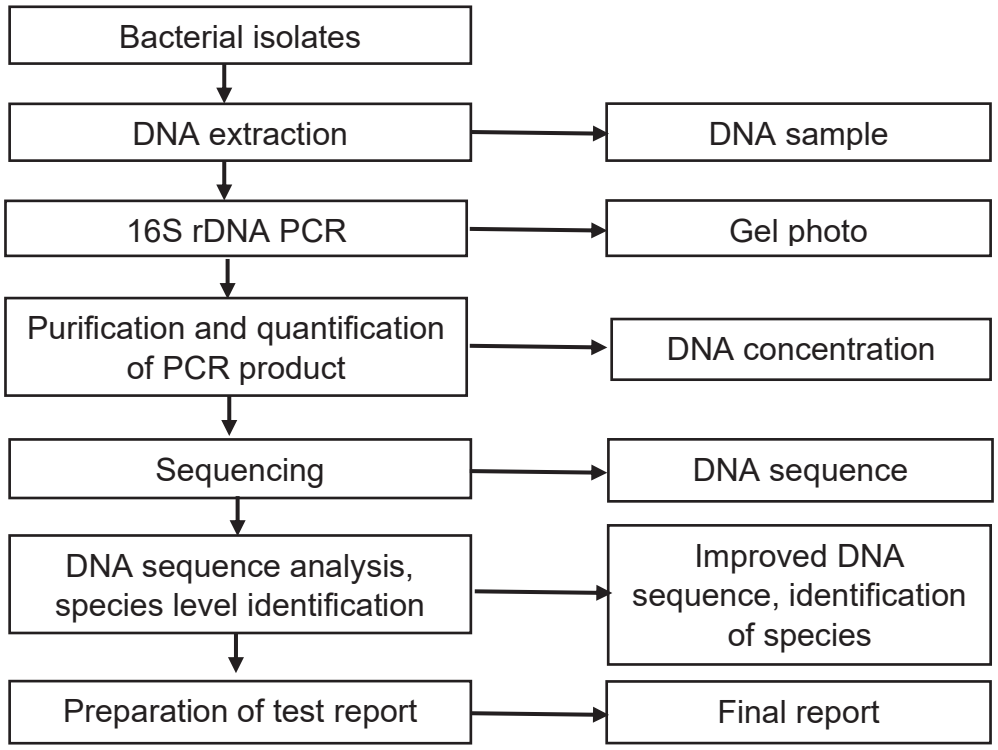

Figure 3. Steps of genetic identification and the result of each step

The seven-step assay method was performed as follows: (1) extraction of genomic DNA; (2) 16S rDNA PCR run; (3) agarose gel electrophoresis; (4) evaluation of gel photo; (5) Purification of PCR product, determination of DNA concentration and purity; (6) DNA sequencing; (7) sequence analysis, evaluation.

3.5.2.1. Extraction of genomic DNA

Glycerol-containing stock solutions of bacterial strains were spread with a loop on MRS agar plates and incubated overnight at 37 °C under anaerobic conditions. Solitary colonies were transferred to MRS agar plates and incubated again. The solitary colonies required for genomic DNA extraction were washed into Eppendorf

tubes filled with 1.5 ml of physiological saline. Genomic DNA purification was performed using the NucleoSpin Microbial DNA kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol **[12].**

3.5.2.2. 16S rDNA PCR

The DNA samples extracted were verified by polymerase chain reaction. For this purpose, a reaction mixture of DreamTaq Green PCR Master Mix (2×), primers 27f and 1492r, AccuGENE water of molecular biological purity (Lonza, Basel, Switzerland) and purified DNA from the bacterial isolates was weighed in a 1.5 ml Safe Seal Eppendorf tube. The assay was performed on a Mastercycler PCR machine (Eppendorf, Hamburg, Germany) running the 16S rDNA program. The specific settings are shown in ***Table 3.***

Table 3. Parameters for each cycle of the 16S rDNS program

Step no.	Name of process	Temperature (°C)	Time	
1	Warm up	95	4 min	
2	Denaturation	95	20 sec	
3	Annealing	54	30 sec	×40*
4	Extension	72	1 min	
5	Elongation	72	5 min	
6	Maintaining temperature	10	∞	

*Steps 2-4 were repeated a total of 40 times.

3.5.2.3. Agarose gel electrophoresis

Upon completion of the program, agarose gel electrophoresis was used to visualize the amplified DNA molecules. To prepare a 1% agarose gel, 0.6 g of agarose (VWR) was dissolved in 60 ml of 1 × TBE (Tris/Borate/EDTA buffer) solution. The mixture was then boiled until the agar was completely dissolved. During cooling after boiling, the solution was stirred with a magnetic stirrer and 6 µl of DNA ECO Safe staining solution (Pacific Image Electronics, Torrance, CA, USA) was added. A gel tray and gel comb were used for gel casting. After solidification of the agarose, the comb was removed from the gel and the tray was placed in the electrophoresis tank, which was filled with buffer (1×TBE solution). PCR reaction products were added to the wells and separation was started.

3.5.2.4. Evaluation of gel photo

After running gel electrophoresis, we checked whether the products all samples contained DNA. To this end, a DNA size marker was added to the gel with the samples before starting the separation.

3.5.2.5 Purification of PCR product, determination of DNA concentration and purity

The DNA concentration and purity of each product were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher) and sent to an external company (Macrogen Europe, Amsterdam, The Netherlands) for sequencing.

3.5.2.6. DNA nucleotide sequence determination (sequencing)

Only a part of the workflow was done in our laboratory located in Mosonmagyaróvár, Hungary. This included the preparation of samples, sending them for sequencing, and the evaluation of results. The DNA samples to be identified were dispensed into 1.5 ml Eppendorf tubes, which were labeled with a sticker bearing the identification number and the name of the primer (i.e., 27f, 1492r). DNA was sequenced with both forward and reverse primers. For this reason, each sample was split in two (5-10 µl). The primers to be used for sequencing were also dispensed and labelled. Finally, all tubes were sealed with parafilm and sent to the service provider (Macrogen Europe).

3.5.2.7. Analysis and evaluation of DNA sequences

The service provider sent the nucleotide sequences of DNA samples via email. The files could be downloaded from the link provided. The Chromas and ChromasPro programs (Technelysium, Brisbane, Australia) were used to manage the files. The sequences obtained with forward and reverse primers were aligned and the non-evaluable bases were trimmed from the beginning and end of the resulting sequence. Where the two sequences differed, the correction offered by the chromatogram was applied. The corrected nucleotide sequence that could be extracted from here was then evaluated using the "nucleotide blast" option of the Basic Local Alignment Search Tool online **[13].**

4. Results and discussion

4.1. Antimicrobial activity tests

The results of tests carried out to determine the antimicrobial activity of our isolates are shown in ***Table 4.***

*Table 4. Results of antimicrobial activity tests (diameter of inhibition zone expressed in mm)**

Inhibitory agent	Treatment**	Bacterial pathogen to be inhibited		
		Escherichia coli **ATCC 8739**	***Staphylococcus aureus*** **ATCC 49775**	***Salmonella*** **Enteritidis ATCC 13076**
Flóraszept	Undiluted	8	14	9.0
Afilact Fluid	Undiluted	0	8	0.0
Isolate E10	K1	0	0	0.0
	K2	0	0	0.0
	K3	0	0	0.0
	K4	0	0	0.0
	K5	0	0	0.0
Isolate E15	K1	0	0	15.3
	K2	0	0	14.2
	K3	0	0	16.5
	K4	0	0	0.0
	K5	0	0	10.9
Isolate E66	K1	0	0	7.3
	K2	0	0	13.8
	K3	0	0	13.7
	K4	0	0	0.0
	K5	0	0	11.7
Isolate E92	K1	0	0	0.0
	K2	0	0	11.7
	K3	0	0	8.0
	K4	0	0	0.0
	K5	0	0	13.0
Isolate E173	K1	0	0	20.8
	K2	0	0	22.3
	K3	0	0	17.5
	K4	0	0	5.8
	K5	0	0	20.5
Isolate E198	K1	0	0	13.0
	K2	0	0	13.8
	K3	0	0	9.3
	K4	0	0	0.0
	K5	0	0	12.2
Isolate E216	K1	0	0	16.0
	K2	0	0	15.0
	K3	0	0	15.0
	K4	0	0	0.0
	K5	0	0	18.0

* Values are means of 9 technical × 2 biological replicates.

** For a detailed description of the treatments, see subsection 3.3.2.3.

ATCC: American Type Culture Collection [a U.S.-based nonprofit organization that provides standard microbial reference strains (Ed.)].

Flóraszept, which served as a positive control, inhibited the growth of all three pathogenic bacterial strains, whereas the lysozyme-containing Afilact Fluid only inhibited the growth of gram-positive *Staph. aureus*, as was expected (***Table 4***).

No treatment of the E10 isolate had an inhibitory effect on the pathogenic bacteria tested. By comparison, Miao et al. **[14]** have reported that *Lacticaseibacillus paracasei* subsp. *tolerans* FX-6 isolated from Tibetan kefir produces a so-called F1 bacteriocin with a broad antimicrobial spectrum. The bacteriocin was found to be heat resistant and its nature did not change at pH values of 3.0, 6.0, and 9.0. A total of 49 lactic acid bacteria strains were isolated by the named authors. The isolates were cultured in sterilized milk and the antimicrobial activity of their cell-free supernatants was tested by the agar well diffusion assay. The cell-free supernatants of seven strains showed an inhibitory activity against *Escherichia coli*. The cell-free supernatants of strains produced an inhibition zone of 10.9±0.1 mm and 12.0±0.4 mm against the indicator *E. coli*. The cell-free supernatant of our isolate E10 was not recovered from milk, but from MRS broth with pH 6.2, and this may have caused the difference between the results of the two studies.

The live cell culture of strain E15 produced an inhibition zone of 15.3 mm in diameter for *Salmonella* Enteritidis ATCC 13076. Interestingly, dead cells of this isolate were also able to inhibit *Salm.* Enteritidis because, despite being heated at 100 °C for 30 min, strain E15 produced an inhibition zone of 14.2 mm. In contrast, the medium adjusted to pH 6.5 had no inhibitory effect on salmonellae, indicating that the antimicrobial agent synthesized by strain E15 is of acidic nature. Centrifugation at 7500 *g* did not result in the extraction of increased levels of antimicrobial substances and an extended inhibition zone.

The viable culture of strain E66 (treatment 1) formed an inhibition zone of only 7.3 mm in diameter, whereas treatments K2 and K3 showed the highest level of inhibition against the *Salmonella* strain tested in this study. Treatment 4 had no detectable effect on any of the pathogenic strains. The intensive treatment 5 did not promote the formation of a larger zone of inhibition.

Neither the live culture nor the neutralized cell-free supernatant of isolate E92 had any detectable inhibitory effect on the pathogenic bacteria tested. Treatment 5 proved to be the most effective, as demonstrated by the 13.0 mm zone of inhibition.

All five treatments of strain E173 inhibited the growth of *Salm.* Enteritidis. The most effective method was heat destruction based treatment 2 with an inhibition zone of 22.3 mm. The cell-free supernatant of this isolate at pH 6.5 slightly inhibited *Salm.* Enteritidis (inhibition zone of 5.8 mm). This demonstrates that strain E173 does not exclusively produce antimicrobials of an acidic nature.

The heat-killed and live cultures of strain E198 produced inhibition zones of 13.8 mm and 13.0 mm, respectively, against *Salm.* Enteritidis. Treatment 4, however, had no detectable effect on any bacterial pathogenic tested.

Unlike treatment 4, treatments 1 and 5 of strain E216 were found to be highly effective against *Salm.* Enteritidis, producing inhibition zones of 16.0 mm and 18.0 mm, respectively.

Overall, none of our lactic acid bacteria isolates had a detectable effect on the tested strains of *E. coli* and *Staph. aureus*. Our results are similar to those of Maragkoudakis et al. **[15]**, who isolated *Lb. acidophilus* ACA-DC 295 from raw milk, *Lcb. paracasei* subsp. *paracasei* ACA-DC 3345 from Cheddar cheese, *Lcb. paracasei* subsp. *tolerans* ACA-DC 4038 from Kasseri cheese, and *Lactiplantibacillus plantarum* ACA-DC 146 from the brine of Feta cheese and tested the antimicrobial activity of these isolates by the agar well diffusion method. It was found that none of the cell-free supernatants of the 29 lactic acid bacteria isolates inhibited the growth of pathogenic *E. coli*, *Salm.* Typhimurium and *Helicobacter pylori* strains.

4.2. Antibiotic resistance testing

Standardized antibiotic resistance studies have suggested that the MRS culture medium commonly used for culturing lactic acid bacteria may have an antagonistic effect against certain antibiotics. The *Lactobacillus* Susceptibility Medium (LSM), consisting of 90% Iso-Sensitest agar and 10% MRS agar, was developed to eliminate these inhibitory effects. LSM has been shown to promote the growth of lactobacilli and bifidobacteria, as recommended by the relevant international standard **[16],** while minimizing potential antagonism between the medium components and the antimicrobials tested **[17]**. Our findings are shown in ***Table 5.***

Table 5. Results of antibiotic susceptibility tests (diameter of inhibition zone expressed in mm)[*]

Strain	Culture medium	Empty disk	Antibiotic[**]										
			CN10	C30	VA30	S10	E15	DA2	AM10	TE30	K30	NA30	SXT25
Lactobacillus acidophilus ATCC 4356	Iso-Sensitest	–											
	MRS	0.0	0.0	25.2 ± 1.6	0.0	8.7 ± 2.3	23.5 ± 0.8	0.0	9.0 ± 3.5	21.0 ± 7.0	0.0	0.0	0.0
	LSM	0.0	11.5 ± 4.6	23.8 ± 1.5	0.0	10.2 ± 4.7	25.5 ± 2.9	0.0	0.0	18.8 ± 6.9	0.0	0.0	0.0
Isolate E10	Iso-Sensitest	0.0	17.0 ± 2.2	20.0 ± 2.3	0.0	13.2 ± 2.7	29.5 ± 3.5	7.7 ± 0.8	0.0	26.7 ± 4.6	2.5 ± 2.1	0.0	4.7 ± 2.7
	MRS	0.0	0.0	25.5 ± 2.1	0.0	0.0	21.2 ± 4.6	11.0 ± 7.6	5.7 ± 1.0	20.3 ± 4.1	0.0	0.0	0.0
	LSM	0.0	12.1 ± 2.8	23.0 ± 0.6	0.0	7.2 ± 2.8	22.3 ± 5.6	12.3 ± 4.5	0.0	21.0 ± 1.9	0.0	0.0	5.5 ± 3.2
Isolate E15	Iso-Sensitest	0.0	17.2 ± 4.1	24.2 ± 4.9	0.0	12.2 ± 0.8	25.2 ± 2.6	0.0	0.0	10.8 ± 2.0	8.5 ± 2.4	0.0	17.0 ± 2.4
	MRS	0.0	2.7 ± 0.5	25.0 ± 0.9	0.0	0.0	20.3 ± 1.9	0.0	0.0	12.5 ± 2.6	0.0	0.0	0.0
	LSM	0.0	14.3 ± 0.5	23.5 ± 2.3	0.0	3.0 ± 0.9	21.8 ± 2.3	0.0	0.0	10.0 ± 2.0	0.0	0.0	22.7 ± 1.2
Isolate E66	Iso-Sensitest	0.0	18.7 ± 0.8	21.2 ± 2.9	0.0	11.7 ± 0.5	25.3 ± 0.8	0.0	0.0	10.8 ± 2.0	8.8 ± 2.1	0.0	16.0 ± 1.3
	MRS	0.0	2.0 ± 0.9	25.2 ± 1.6	0.0	0.0	22.2 ± 1.6	0.0	5.7 ± 1.2	14.3 ± 0.8	2.3 ± 0.5	0.0	0.0
	LSM	0.0	14.5 ± 1.5	21.8 ± 3.4	0.0	4.3 ± 2.1	22.2 ± 1.5	0.0	0.0	12.7 ± 0.8	2.5 ± 1.0	0.0	22.7 ± 1.2
Isolate E92	Iso-Sensitest	0.0	17.1 ± 2.7	19.4 ± 3.8	0.0	8.6 ± 2.7	18.2 ± 4.6	0.0	8.6 ± 4.0	8.3 ± 1.7	6.0 ± 1.9	0.0	14.3 ± 3.3
	MRS	0.0	7.3 ± 1.0	21.4 ± 2.2	0.0	1.0 ± 0.1	17.6 ± 1.9	0.0	10.2 ± 1.8	10.2 ± 2.9	0.0	0.0	0.0
	LSM	0.0	13.9 ± 2.4	15.1 ± 2.0	0.0	5.6 ± 1.5	18.7 ± 2.6	0.0	7.7 ± 2.5	8.4 ± 1.8	2.9 ± 2.3	0.0	20.7 ± 4.5
Isolate E173	Iso-Sensitest	0.0	15.4 ± 1.4	22.6 ± 1.1	0.0	7.3 ± 2.2	21.7 ± 5.2	0.0	7.8 ± 3.6	10.7 ± 1.2	5.1 ± 0.8	1.8 ± 1.1	16.3 ± 2.4
	MRS	0.0	5.9 ± 1.1	23.4 ± 2.0	0.0	1.3 ± 0.7	22.8 ± 2.3	0.0	8.7 ± 3.7	11.7 ± 4.2	0.0	0.0	0.0
	LSM	0.0	12.9 ± 2.3	21.7 ± 2.2	0.0	5.2 ± 0.8	21.6 ± 2.3	0.0	17.6 ± 2.1	10.8 ± 0.8	0.0	4.2 ± 4.4	21.6 ± 3.6
Isolate E198	Iso-Sensitest	0.0	15.0 ± 2.2	19.8 ± 2.9	0.0	6.4 ± 0.7	20.4 ± 3.0	0.0	8.7 ± 3.2	9.8 ± 2.0	2.7 ± 0.7	0.0	21.9 ± 1.6
	MRS	0.0	7.2 ± 1.8	21.0 ± 2.1	0.0	0.0	18.2 ± 2.7	0.0	10.8 ± 3.5	10.8 ± 2.4	0.0	0.0	0.0
	LSM	0.0	13.6 ± 1.8	20.0 ± 2.3	0.0	4.8 ± 1.7	19.9 ± 4.1	0.0	7.2 ± 3.0	9.9 ± 2.5	1.8 ± 0.7	0.0	22.3 ± 1.4
Isolate E216	Iso-Sensitest	0.0	14.8 ± 2.0	23.1 ± 1.5	0.0	8.8 ± 2.0	20.2 ± 1.9	6.7 ± 1.3	9.1 ± 5.0	12.4 ± 1.0	5.3 ± 2.6	0.0	17.2 ± 2.5
	MRS	0.0	3.0 ± 1.7	21.8 ± 3.3	0.0	0.0	17.8 ± 2.8	7.8 ± 1.4	20.9 ± 2.9	11.1 ± 1.3	0.0	0.0	12.0 ± 2.1
	LSM	0.0	11.7 ± 1.2	22.4 ± 1.7	0.0	4.3 ± 1.1	19.1 ± 1.8	7.8 ± 1.2	13.1 ± 3.6	11.6 ± 2.4	0.0	0.0	18.0 ± 1.7

[*] Values are means ± SD of 3 technical × 2 biological replicates.
[**] CN10: 10 µg gentamicin, C30: 30 µg chloramphenicol, VA30: 30 µg vancomycin, S10: 10 µg streptomycin, E15: µg erythromycin, DA2: 2 µg clindamycin, AM10: 10 µg ampicillin, TE30: 30 µg tetracycline, K30: 30 µg kanamycin, NA30: 30 µg nalidixic acid, SXT25: 1.25 µg trimethoprim + 23.75 µg sulfamethoxazole.

Both *Lb. acidophilus* ATCC 4356 and our pre-selected isolates were found to be resistant to vancomycin and nalidixic acid, which is explained by the report of Charteris and et al. **[18]** that *Lactobacillus* species are inherently resistant to nalidixic acid. Wolupeck et al. **[19],** studying the antibiotic resistance of lactobacilli, found that all of their strains were resistant to ciprofloxacin, gentamicin, streptomycin, and vancomycin, but were sensitive to tetracycline and chloramphenicol. Our isolates (and *Lb. acidophilus* ATCC 4356) were similarly sensitive to the latter two antibiotics (***Table 5***).

It is worth mentioning that *Lb. acidophilus* ATCC 4356 could not grow in Iso-Sensitest agar (***Table 5***). Huys et al. **[17]** also concluded that Iso-Sensitest agar cannot be recommended for use in antibiotic susceptibility testing of lactic acid bacteria.

4.3. Genetic identification

The results of genetic identification are summarized in ***Table 6.*** All seven strains isolated from Transylvanian raw milk, fermented milk, and cheese samples were found to belong to lactobacilli **[11]**. Sequencing resulted in the identification of one *Lcb. paracasei* subsp*. tolerans*, five *Levilactobacillus brevis*, and one *Lpb. plantarum* with an accuracy percentage of 99-100.

Table 6. Results of genetic identification

Isolate	Number of identical / total nucleotides	Number of		Identified strain
		differing	missing	
		base pairs		
E10	1070 / 1070 (100%)	0	0	*Lacticaseibacillus paracasei* subsp*. tolerans* NBRC 15906
E15	1199 / 1200 (99,9%)	1	0	*Levilactobacillus brevis* ATCC 14869
E66	1194 / 1194 (100%)	0	0	*Levilactobacillus brevis* ATCC 14869
E92	1118 / 1120 (99,8%)	2	0	*Levilactobacillus brevis* ATCC 14869
E173	995 / 999 (99,6%)	4	3	*Levilactobacillus brevis* ATCC 14869
E198	1185 / 1198 (98,9%)	13	11	*Levilactobacillus brevis* ATCC 14869
E216	1102 / 1102 (100%)	0	0	*Lactiplantibacillus plantarum* JCM 1149

5. Conclusions

Overall, our efforts to develop and apply additional elements of an *in vitro* test system for the selection of probiotic strains have been successful. Further refinement of the individual steps may not be necessary, as they are already suitable for pre-selection of even high numbers of isolates. If the strains are to be used as probiotics, it may be worthwhile to test their inhibitory activity on a number of other pathogenic microbes and to explore the presence of transferable antibiotic resistance genes by molecular biology methods. Using all the elements of the *in vitro* test system, we have found no isolates that could safely be used for the manufacture of probiotic products. However, our test system has been shown to be capable of effectively screening out unsafe isolates.

6. Acknowledgments

Zoltán Hatvan is grateful for the financial support of the New National Excellence Program of the Ministry of Innovation and Technology (project ID: ÚNKP-21-2-I-SZE-32).

The authors thank the Recovery and Resilience Facility and the National Recovery Fund for supporting this study (project no.: RRF-2.3.1-21-2022-00001).

7. References

[1] Fuller, R. (1989): Probiotics in man and animals. Journal of Applied Bacteriology 66 pp. 365-378. https://doi.org/10.1111/j.1365-2672.1989.tb05105.x

[2] Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L., Canani, R.B., Flint, H.J., Salminen, S., Calder, P.C., Sanders, M.E. (2014): The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature Reviews Gastroenterology and Hepatology 11 pp. 506-514. https://doi.org/10.1038/nrgastro.2014.66

[3] Fijan, S., Frauwallner, A., Varga, L., Langerholc, T., Rogelj, I., Lorber, M., Lewis, P., Povalej-Bržan, P. (2019): Health professionals' knowledge of probiotics: an international survey. International Journal of Environmental Research and Public Health 16 3128. https://doi.org/10.3390/ijerph16173128

[4] Fijan, S., ter Haar, J.A., Varga, L. (2021) Probiotic microorganisms and their benefit to human health. In Advances in Probiotics: Microorganisms in Food and Health, eds. Dhanasekaran, D., Sankaranarayanan, A. pp. 3-22. Academic Press and Elsevier: London, San Diego, Cambridge, Oxford. https://doi.org/10.1016/B978-0-12-822909-5.00001-0

[5] Süle, J., Varga, L., Varga, K., Hatvan, Z., Kerényi, Z. (2022) Probiotikus baktériumtörzsek szelektálására alkalmas kísérleti rendszer egyes elemeinek kidolgozása (Developing basic elements of an experimental system for selection of probiotic bacterial strains). Magyar Állatorvosok Lapja 144 (közlésre benyújtva).

[6] Papadimitriou, K., Zoumpopoulou, G., Foligné, B., Alexandraki, V., Kazou, M., Pot, B., Tsakalidou, E. (2015): Discovering probiotic microorganisms: *in vitro, in vivo*, genetic and omics approaches. Frontiers in Microbiology 6 58. https://doi.org/10.3389/fmicb.2015.00058

[7] Williams, C.F., Walton, G.E., Jiang, L., Plummer, S., Garaiova, I., Gibson, G.R. (2015): Comparative analysis of intestinal tract models. Annual Review of Food Science and Technology 6 pp. 329-350. https://doi.org/10.1146/annurev-food-022814-015429

[8] Antal, O., Némethné Szerdahelyi, E., Takács, K. (2020): *In vitro* humán emésztési modellek alkalmazása a táplálkozástudomány területén (Application of *in vitro* human digestion models in the field of nutrition science). Élelmiszervizsgálati Közlemények - Journal of Food Investigation 66 pp. 3141-3157.

[9] Süle, J., Varga, L., Hatvan, Z., Kerényi, Z. (2022) *In vitro* tesztrendszer alkalmazása probiotikus baktériumtörzsek szelektálására (Application of an *in vitro* test system for the selection of probiotic bacterial strains). Élelmiszervizsgálati Közlemények - Journal of Food Investigation 68 pp. 3904-3927.

[10] Ouwehand, A.C., Forssten, S., Hibberd, A.A., Lyra, A., Stahl, B. (2016): Probiotic approach to prevent antibiotic resistance. Annals of Medicine 48 pp. 246-255. https://doi.org/10.3109/07853890.2016.1161232

[11] Zheng, J.S., Wittouck, S., Salvetti, E., Franz, C.M.A.P., Harris, H.M.B., Mattarelli, P., O'Toole, P.W., Pot, B., Vandamme, P., Walter, J., Watanabe, K., Wuyts, S., Felis, G.E., Gänzle, M.G., Lebeer, S. (2020): A taxonomic note on the genus *Lactobacillus*: description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. International Journal of Systematic Evolutionary Microbiology 70 pp. 2782-2858. https://doi.org/10.1099/ijsem.0.004107

[12] URL[1]: https://www.mn-net.com/media/pdf/91/f9/1f/Instruction-NucleoSpin-Microbial-DNA.pdf (Hozzáférés: 2022. 05. 03.)

[13] URL[2]: https://blast.ncbi.nlm.nih.gov/Blast.cgi (Hozzáférés: 2022. 04. 11.)

[14] Miao, J.Y., Guo, H.X., Ou, Y.W., Liu, G., Fang, X., Liao, Z.L., Ke, C., Chen, Y.J., Zhao, L.C., Cao, Y. (2014): Purification and characterization of bacteriocin F1, a novel bacteriocin produced by *Lactobacillus paracasei* subsp. *tolerans* FX-6 from Tibetan kefir, a traditional fermented milk from Tibet, China. Food Control 42 pp. 48-53. https://doi.org/10.1016/j.foodcont.2014.01.041

[15] Maragkoudakis, P.A., Zoumpopoulou, G., Miaris, C., Kalantzopoulos, G., Pot, B., Tsakalidou, E. (2006): Probiotic potential of *Lactobacillus* strains isolated from dairy products. International Dairy Journal 16 pp. 189-199. https://doi.org/10.1016/j.idairyj.2005.02.009

[16] International Organization for Standardization (ISO), International Dairy Federation (IDF) (2010): Milk and milk products – Determination of the minimal inhibitory concentration (MIC) of antibiotics applicable to bifidobacteria and non-enterococcal lactic acid bacteria (LAB). International Standard ISO 10932:2010(E) and IDF 223:2010(E). ISO, Geneva, Switzerland and IDF, Brussels, Belgium.

[17] Huys, G., D'Haene, K., Swings, J. (2002): Influence of the culture medium on antibiotic susceptibility testing of food-associated lactic acid bacteria with the agar overlay disc diffusion method. Letters in Applied Microbiology 34 pp. 402-406. https://doi.org/10.1046/j.1472-765X.2002.01109.x

[18] Charteris, W.P., Kelly, P.M., Morelli, L., Collins, J.K. (2001): Gradient diffusion antibiotic susceptibility testing of potentially probiotic lactobacilli. Journal of Food Protection 64 pp. 2007-2014. https://doi.org/10.4315/0362-028X-64.12.2007

[19] Wolupeck, H.L., Morete, C.A., DallaSanta, O.R., Luciano, F.B., Madeira, H.M.F., de Macedo, R.E.F. (2017): Methods for the evaluation of antibiotic resistance in *Lactobacillus* isolated from fermented sausages. Ciência Rural 47 e20160966. https://doi.org/10.1590/0103-8478cr20160966