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Application of an in vitro test system for the selection of probiotic bacterial strains

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1. SUMMARY

The aim of our studies was to evaluate *in vitro* methods for the simple and efficient selection of putative probiotic bacterial strains. Of the possible methods, the following were tested: culturing on selective media, Gram staining, catalase assay, hemolytic, clonality and aggregation ability, gastric acid tolerance and bile acid tolerance. A total of 217 bacterial strains isolated from raw sheep's milk, curdled milk and sheep's cheese samples produced in Transylvania were included in our experiments. Isolates with hemolytic activity, as well as those exhibiting Gram-negative or catalase-positive phenotypes not characteristic of probiotics were excluded from our studies. Based on the results of RAPD-PCR studies suitable for the detection of individual-level polymorphisms, a total of 34 clone classes and 57 strains with unique RAPD patterns were identified. From each of the 34 clone classes thus narrowed, one strain was selected and tested for its aggregation ability, as well as its gastric acid and bile acid tolerance. High aggregation values above 70%, typical of probiotic strains, were measured in the case of a total of six isolates. In the course of the presence-absence studies conducted on the surface of solid media supplemented with acid or bile acid, it was possible to select several strains specifically tolerant to acid or bile acid. Based on our results, isolates to be included in further tests, e.g., in antibiotic resistance and antimicrobial activity assays, were selected.

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2. Introduction

Probiotics are living organisms that, when used in appropriate amounts, have a beneficial effect on the health of the host organism **[1, 2]**. They must meet a number of conditions in order to be allowed to display the probiotic designation. Among other things, they need to have an increased tolerance to various body fluids (gastric acid, bile acids, digestive enzymes) and they must stabilize the intestinal microbiota by binding to intestinal epithelial cells through their ability to adhere **[3]**.

There is a growing worldwide demand for probiotic products that have a beneficial effect on health, both in terms of human consumption and the feeding of farm animals. The use of antibiotics for yield enhancement has been banned in the European Union since 2006 **[4],** and the focus has been even more on probiotics since that date.

Year after year, a large number of bacterial strains are isolated in order to exploit their beneficial effects on health. Complex and costly animal studies must be preceded by a selection system of in vitro experiments that allows the simple, rapid and cost-effective selection of strains, from hundreds or even thousands of isolates, that will hopefully prove probiotic in in vivo studies **[5, 6, 7]**.

Based on the above, the aim of our work was to develop and evaluate in vitro measurement methods that can be used to quickly and efficiently investigate the classical microbiological characteristics, clonality, aggregation ability, as well as the resistance to gastric acid and bile acid of bacterial strains. We sought to answer the question whether the large number of isolates studied by us included clones and strains with potentially beneficial (even probiotic) properties that should be included in further in vitro studies. It was also examined whether the test system was working well or whether it was necessary to optimize the individual steps, as well as what acid and bile acid concentrations the different strains were able to tolerate, and whether there was any correlation between the results of the aggregation test and the acid and bile acid tolerance. Accordingly, of the in vitro tests, the results obtained by the following test methods are presented in our publication:

- Classical microbiological tests (culturing on selective media and determination of colony morphology, Gram staining and subsequent microscopic examination, catalase test, hemolysis test)
- Clonality assay by RAPD-PCR method
- Autoaggregation test
- Presence-absence studies conducted on the surface of solid media supplemented with acid or bile acid

3. Materials and methods

3.1. Isolation, culturing, preservation and storage of bacterial strains

Our studies were conducted with bacterial strains isolated from raw sheep's milk, curdled milk and sheep's cheese samples produced in Transylvania. The products had a natural microbiota and no commercially available starter cultures were used for their production. For the preparation of the cheeses, rennet was made by the shepherds from veal stomachs. The goal was to isolate highly efficient probiotic strains that would be later used in the development of probiotic products. Restoration and culturing of the 217 isolates and the control strains were performed under the conditions listed in ***Table 1***.

Table 1. Restoration and culturing conditions of the isolated and control bacterial strains included in the experiments

Strain	Broth	Culture medium	Incubation conditions
Lactobacillus acidophilus ATCC 4356	MRS broth (pH = 6.2)	MRS agar (pH = 6.2)	37 °C, 72 hours, anaerobic
Lactobacillus acidophilus LA-5	MRS broth (pH = 6.2)	MRS agar (pH = 6.2)	37 °C, 72 hours, anaerobic
Staphylococcus aureus subsp. aureus ATCC 49775	CASO broth	CASO agar	37 °C, 24 hours, aerobic
E1–E92 isolates	MRS broth (pH = 6.2)	MRS–CC agar*	37 °C, 72 hours, anaerobic
E93–E217 isolates	MRS broth (pH = 5.4)	MRS agar (pH = 5.4)	37 °C, 72 hours, anaerobic

* De Man–Rogosa–Sharpe agar supplemented with clindamycin and ciprofloxacin

Isolates were preserved and stored in glycerol stock solutions. A strain taken from the surface of MRS–CC agar or MRS pH 5.4 agar was washed into 3 ml of broth with an inoculating loop, and then it was incubated according to the needs of the strain. 300 µl of the grown culture was added to a cryo (freezer) tube, 900 µl of a 60% glycerol solution was added, it was vortexed and frozen in liquid nitrogen for ca. 30 seconds. Storage was conducted at -80 °C in an ultra-freezer.

3.2. Selective culture conditions, its media and their preparation

3.2.1. Physiological saline solution

For the preparation of the diluent used to prepare the decimal dilution series, 8.5 g of NaCl and 1 g of tryptone were weighed and dissolved in 1 L of distilled water. 9.3 ml was added to the test tubes, and they were sterilized in an autoclave at 121 °C for 15 minutes.

3.2.2. Phosphate buffer solution (PBS)

For one liter of distilled water, the following substances were weighed on an analytical balance: 80 g of sodium chloride, 2 g of potassium chloride, 14.4 g of disodium hydrogen phosphate dodecahydrate and 2.4 g of potassium dihydrogen phosphate. Dissolution was aided by a magnetic stirrer and when the solution became particle-free, it was sterilized in an autoclave at, 121 °C for 15 minutes. The solution thus prepared corresponds to PBS with a tenfold concentration, i.e., for further use it has to be diluted as follows: 100 ml of 10× PBS solution is added to 900 ml of distilled water. After proper mixing, the 1× PBS solution is ready for use.

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

Of commercially available MRS broth (VWR, Radnor, PA, USA) or MRS agar (VWR), the quantity recommended by the manufacturer was weighed to analytical accuracy and ten dissolved in distilled water, using a magnetic stirred until dissolved. The pH was adjusted to the desired value (6.2 ± 0.2) with 1 M HCl. Following this, the culture media were sterilized in an autoclave at 121 °C for 15 minutes.

3.2.4. MRS agar (pH = 5.4)

MRS agar (VWR) was prepared according to the manufacturer's instructions, its pH value was adjusted to 5.4 with 1 M HCl, and then it was sterilized in an autoclave under standard conditions (121 °C, 15 minutes).

3.2.5. MRS agar supplemented with clindamycin and ciprofloxacin (MRS–CC)

In addition to the basic MRS agar, MRS–CC agar also contained two antibiotic stock solutions that could not be sterilized in an autoclave. For the preparation of one of the stock solutions, 2.0 mg of clindamycin hydrochloride (Sigma Aldrich, St. Louis, MO, USA) was dissolved in 10 ml of distilled water, while for the other, 20.0 mg of ciprofloxacin hydrochloride (Sigma Aldrich) was dissolved in 10 ml of distilled water. The antibiotic stock solutions were then filtered through a 0.22 µm pore size membrane filter (Millipore, Burlington, MA, USA) into sterile screw-capped Erlenmeyer flasks. 0.1 ml of clindamycin and 1.0 ml of ciprofloxacin stock solutions were added to the MRS agar cooled to 45 °C under aseptic conditions, using sterile, disposable pipettes (Greiner Bio-One Hungary, Mosonmagyaróvár, Hungary). Thus, the final concentration of clindamycin in the basic MRS agar was 0.1 mg/l, while that of ciprofloxacin was 10.0 mg/l.

3.2.6. CASO agar

CASO agar (VWR) and CASO broth (VWR) were prepared according to the manufacturer's instructions. Sterilization was performed in an autoclave under standard conditions, at 121 °C for 15 minutes.

3.2.7. Anaerobic culturing

Anaerobic conditions in the course of our studies were ensured as follows: agar plates were incubated in an AnaeroPack Rectangular jar (Merck, Darmstadt, Germany), with the addition of GENbox anaer anaerobic salt (bioMériux, Marcy-l'Étoile, France). Information on the existence of anaerobic conditions was provided by the color change of the Microbiologic Aerotest indicator (Merck) from white to blue.

3.3. Classical microbiological tests

3.3.1. Examination of colony morphology

Macromorphological characteristics of the restored strains were recorded. Among other things, the size, color, surface properties (glossy, matte) of the colonies, as well as the design of the edges of the colonies (regular, irregular, jagged) were observed.

3.3.2. Gram staining

One drop of distilled water, in which a solitary colony was suspended, was added to a degreased slide. The dried smear was stained with crystal violet solution for 2 minutes, then it was treated with lugol solution for 1 minute. Following this, the sample was rinsed with distilled water, then treated with a decolorizing solution for half a minute, which extracted the dye from the Gram-negative cells but not the Gram-positive ones. After another rinsing with distilled water, contrast staining was carried out with safranin for 1 minute. This was followed by rinsing with distilled water, the smears were allowed to dry, and then they were examined under a light microscope (Axio Scope, Carl Zeiss, Oberkochen, Germany) at various magnifications. Performing Gram staining is important because lactic acid bacterial strains with potential probiotic properties are among Gram-positive microbes.

3.3.3. Catalase test

There are microorganisms that produce catalase enzymes that can break down toxic hydrogen peroxide into water and oxygen ($2\ H_2O_2 = 2\ H_2O + O_2$). In order to confirm the catalase production of our isolates, colonies of fresh cultures were placed on slides using an inoculation loop, and a drop of 3% H_2O_2 was added. In positive cases, colonies began to visibly bubble. S. aureus strain ATCC 49775 was used as a positive control, which indicated catalase activity with strong effervescence. Catalase-positive strains are not suitable as probiotics for sure.

3.3.4. Hemolysis test

In the coarse of our hemolysis studies, one colony of each freshly restored strain was transferred to Columbia blood agar (Biolab Zrt., Budapest, Hungary). Results were evaluated after 24 hours of anaerobic incubation at 37 °C. S. aureus, which exhibits β-hemolysis on 5% sheep blood culture medium, was again used as a positive control.

3.4. Clonality test

Bacterial DNA was isolated from the bacterial strains using Chelex 100 Resin (Bio-Rad, Hercules, CA, USA), according to the protocol provided by the manufacturer. For the polymerase chain reaction, the reaction mixture containing the Red Taq 2 mM $MgCl_2$ Master Mixet (VWR), the primer named 1254 chosen by us (Bio-Science, Budapest, Hungary), molecular biology grade AccuGENE water (Lonza, Basel, Switzerland) and the sample (DNA template of the bacterial strains) were measured into a 1.5 ml Eppendorf tube. The samples were analyzed by RAPD-PCR, using the RAPD_03 program of a Mastercycler PCR (Eppendorf, Hamburg, Germany) instrument, the parameters of which are shown in ***Table 2***.

Table 2. Parameters of the RAPD-PCR method

Step	Temperature (°C)	Time	
1.	95	2 min	
2.	94	20 sec	
3.	38	20 sec	x40
4.	72	1 min	
5.	72	5 min	
6.	10	∞	

Steps 2 through 4 were carried out 40 times. Following the completion of the program, the amplified DNA molecules were made visible and evaluated by gel electrophoresis. A 1% agarose gel was prepared for the gel electrophoresis. 0.6 g of agarose (VWR) was weighed and dissolved in 60 ml of 1×TBE TRIS-boroacetic acid solution. The solution was boiled until completely homogenized. It was cooled to lukewarm temperature and 6 µl of DNS ECO Safe dye solution (Pacific Image Electronics, Torrance, CA, USA) was added. Meanwhile, it was agitated on a magnetic stirrer, and then the gel was poured. The cooled gel with the dye was poured into the tray. After setting, the tray was placed in the electrophoresis tank, previously filled with gel electrophoresis buffer (1×TBE solution), then the gel comb was removed. The RAPD-PCR reaction products were then added to the individual pockets.

3.5. Investigation of autoaggregation

The test method used was based on the research of Del Re et al. **[8]**, with minor modifications. Our own isolates and control strains were incubated at 37 °C for 18 hours under anaerobic conditions in MRS broth at pH 6.2. The samples were then centrifuged (Eppendorf Centrifuge 5804 R) at 2426 × g for 6 minutes.

The supernatant was discarded, 50 ml of 1×PBS solution was measured onto the bacterial pellets, and they were vortexed (10 sec). They were centrifuged again the supernatant was discarded and the pellet was redissolved in 1×PBS solution. After vortexing, 900 µl of 1×PBS and 100 µl of cell suspension were measured into semi-micro cuvettes (Greiner Bio-One Hungary). Optical density was measured at a wavelength of 600 nm with a BioMate 160 UV-VIS spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA), and the OD_{600} values were standardized to 0.2 for each sample for the measurement results to be comparable. The set values were checked by OD_{600} measurements. In the case of appropriate values, 4 ml each of bacterial suspension was dispensed into sterile Wassermann tubes, labeled A, B and C for each sample, to ensure three technical replicates. The samples thus prepared were aerobically incubated in Wassermann tubes at room temperature during the assay. Optical density measurements were performed at 0, 5 and 24 hours. At each measurement time point, 200 µl was removed from the top of the bacterial suspension with a wide-tip pipette tip (Axygen, Union City, CA, USA), and it was diluted with 800 µl of 1×PBS solution in a semi-micro cuvette. At each of the three measurement times, the OD_{600} value of each lettered sample was measured three times and the percentage of aggregation was calculated according to the formula given by García-Cayuela et al. **[9]**

$$[1 - (A_{measurement\ time} / A_0) \times 100],$$

where: $A_{measurement\ time}$: the absorbance value of the cell suspension at the given measurement time (5 h, 24 h); A_0: the absorbance value of the cell suspension at time 0 h.

Currently, there is no uniform system for the assessment of autoaggregation. In the course of their studies, Del Re et al. **[8]** rated strains with an aggregation value of >80% as well aggregating isolates, while strains with a value of <10% were considered non-aggregating.

3.6. Analysis of acid and bile acid tolerance

3.6.1. Acid and bile acid culture media required for the test

To test for acid tolerance, MRS culture medium (VWR) was prepared as described, and it was sterilized in an autoclave at 121 °C for 15 minutes. Next, the pH was adjusted with 1 M HCl under aseptic conditions to the following values: 6.0; 5.5; 5.0; 4.0; 3.0. The sterile culture media were cooled back to 45 °C, and plates were poured into square Petri dishes (Greiner Bio-One Hungary). The MRS culture medium with a pH of 6.0 served as the untreated medium.

Too test for bile acid tolerance, the MRS culture medium (VWR) was prepared according to the manufacturer's instructions. After sterilization (at 121 °C, 15 min), sterile-filtered porcine bile (Sigma Aldrich) was added to the basic agar cooled back to 45 °C, using a 0.45 µm pore size membrane filter (Thermo Fisher Scientific). Supplementation was performed to achieve final bile concentrations of 0%, 0.1%, 0.2% and 0.5% in the culture medium. MRS agar containing no bile served as a negative control.

3.6.2. Strain restoration and optical density (OD) measurement

Bacterial strains were restored in a pH 6.2 MRS broth as a result of anaerobic incubation at 37 °C for 18 hours. The multiplied cultures formed more or less pellets at the bottom of the Falcon tube, which was evaluated. The cultures were centrifuged (2426 × g, 6 min, room temperature) (Eppendorf Centrifuge 5804 R). The supernatant was discarded, and the samples were redissolved in 1×PBS solution. After a short (10 sec) vortexing, centrifugation was repeated, and the supernatant was discarded again. After redissolution in 1×PBS solution, vortexing was performed for 10 sec, and the optical density of a 10-fold dilution of the suspension was measured with a BioMate 160 UV-VIS spectrophotometer (Thermo Fisher Scientific) at 600 nm. Following the measurement, suspensions with a uniform OD_{600} value of 0.5 were prepared. For accuracy, the OD_{600} values of the suspensions with adjusted cell densities were remeasured.

3.6.3. Presence-absence test

Of the cell suspensions with an OD_{600} = 0.5, 18 (9 technical × 2 biological replicates) × 10 µl were applied to the surface of culture media with different pH values and bile acid contents, and then the plates were incubated at 37 °C for 48 hours, as described in Section 3.2.7.

3.6.4. Process of bile acid and hydrochloric acid treatment

The tested bacterial strains were treated with bile acid and hydrochloric acid, according to the agents added to the culture media. MRS culture media with a pH of 6.0 with no bile or hydrochloric acid served as negative controls. The procedure of the tests is illustrated in ***Figure 1***.

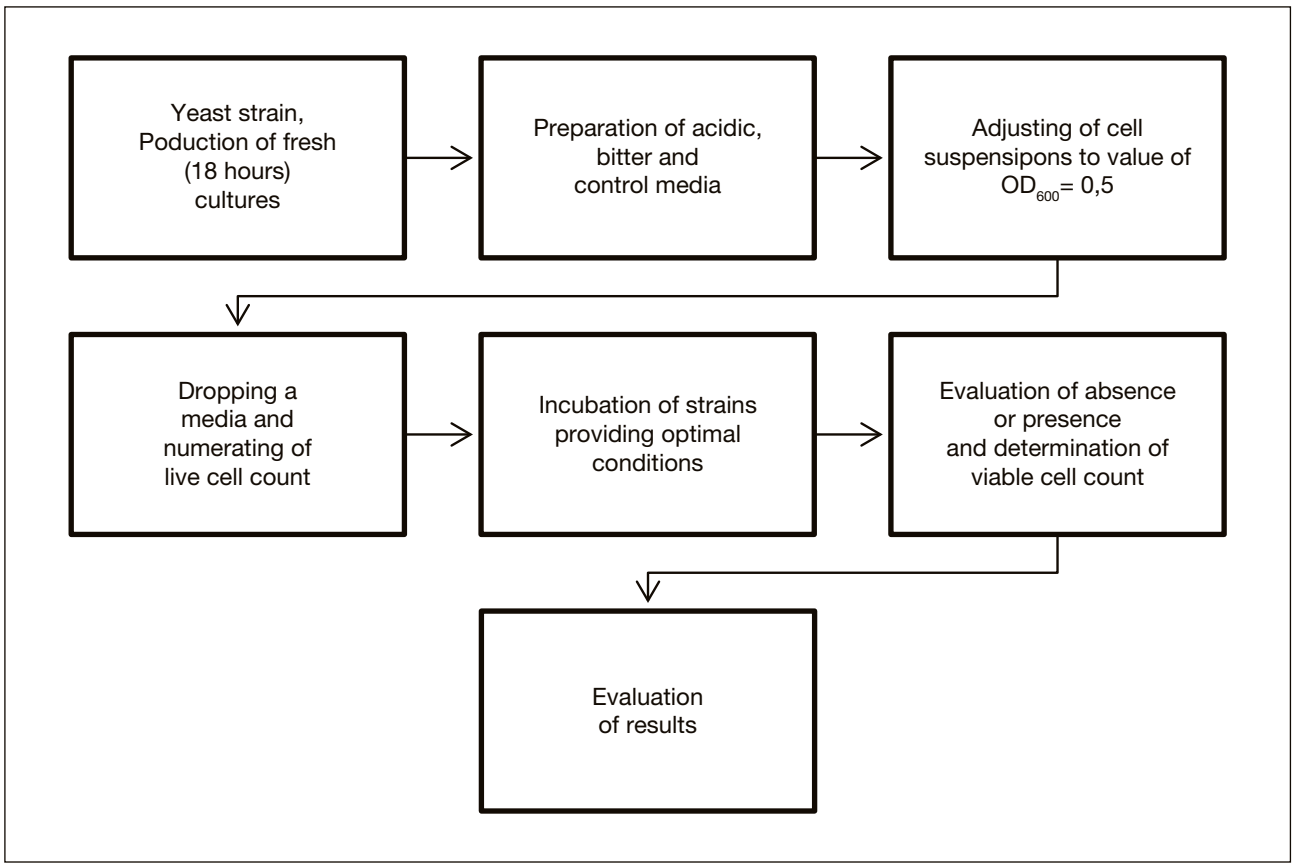

Figure 1. Flow chart of bile acid and hydrochloric acid treatment

3.6.5. Inoculation and viable cell count determination

Decimal dilution series were prepared from the cultures of both our own isolates and the control bacterial strains, and then 100 µl of each dilution member was spread on the surface of MRS agar plates with a pH value of 6.0. The plates thus prepared were incubated at 37 °C for 72 hours under anaerobic conditions. At the end of the incubation period, the colonies were counted.

4. Results and evaluation

4.1. Classical microbiological tests

The aim of classical microbiological tests was to select Gram-negative, catalase-positive and hemolyzing strains. Using these methods, we were able to eliminate out of the 217 isolates those that did not meet the criteria for probiotics. ***Table 3*** shows a non-exhaustive list of strains with appropriate characteristics based on the results of classical microbiological tests, which were included in subsequent studies (aggregation, acid tolerance and bile acid tolerance studies).

Table 3. Main characteristics of strains based on the results of classical microbiological tests

Strain	Colony morphology*	Gram staining	Catalase test	Hemolysis test
E10	Tiny, white, irregular colony with creamy surface	Gram+ rods	Negative	Negative
E15	Large, white, snowflake-like colony	Gram+ rods	Negative	Negative
E66	Large, white, irregular, matte colony with jagged edge	Gram+ rods	Negative	Negative
E92	Large, white, regular, matte colony	Gram+ rods	Negative	Negative
E173	Large, white, irregular, matte colony with jagged edge	Gram+ rods	Negative	Negative
E198	Large, white, buttery, irregular, matte colony	Gram+ rods	Negative	Negative
E216	Tiny, white, buttery, irregular colony with creamy surface	Gram+ rods	Negative	Negative
LA-5	Large, white, regular colony with creamy surface	Gram+ rods	Negative	Negative

*Colony morphology was examined with strains developed on MRS agar adjusted to a pH value of 6.2.

Of the 217 isolates, 25 catalase-positive and 29 Gram-negative strains were identified. These were also excluded from the clone classes and from individual strains that did not fit into the clone classes after the clonality test. None of the strains hemolyzed on blood agar, so although this test did not help to narrow down the large sample number, it was absolutely necessary to perform it to assess the safety of the probiotic strains.

According to the practice of our group, Sedlačková et al. Also included only Gram-positive, rod-shaped and catalase-negative isolates in their further in vitro studies ***[10]***. In their study, a total of 59 Gram-positive and catalase-negative strains were isolated, of which 7 were isolated from raw milk and 12 from cheese prepared from raw cow's milk. The colony morphology was found to be similar to that of the colonies of *L. acidophilus LA-5*.

4.2. Clonality test

RAPD-PCR assays were carried out in parallel with classical microbiological tests. Based on the unique RAPD patterns, the 217 strains were classified into 34 clone classes, of which a gel photograph of clone class 34 is shown in ***Figure 2; Figure 3*** shows several clone classes and individual strains.

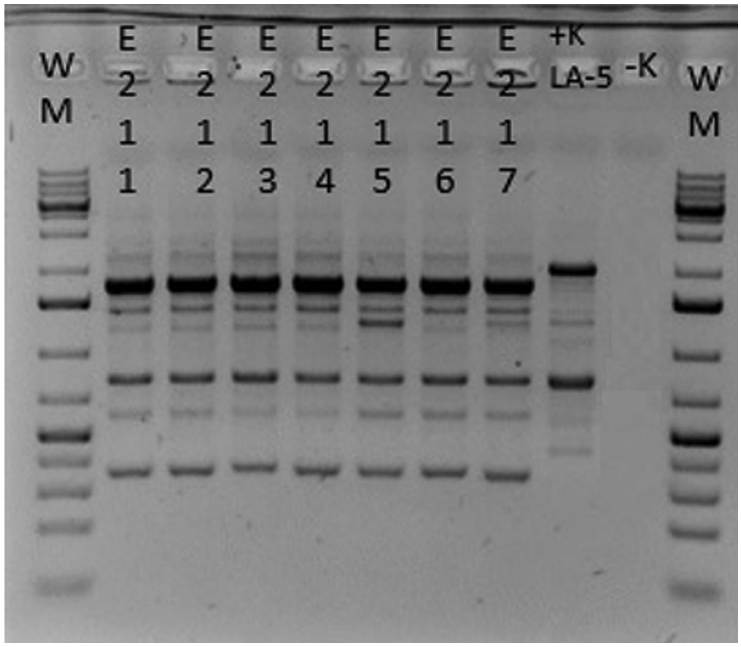

Figure 2. Clonality test of members of clone class 34 (samples: E211–E217, positive control: Lactobacillus acidophilus LA-5, negative control: distilled water, molecular marker: WM; Gene Ruler 1 kb Plus DNA Ladder)

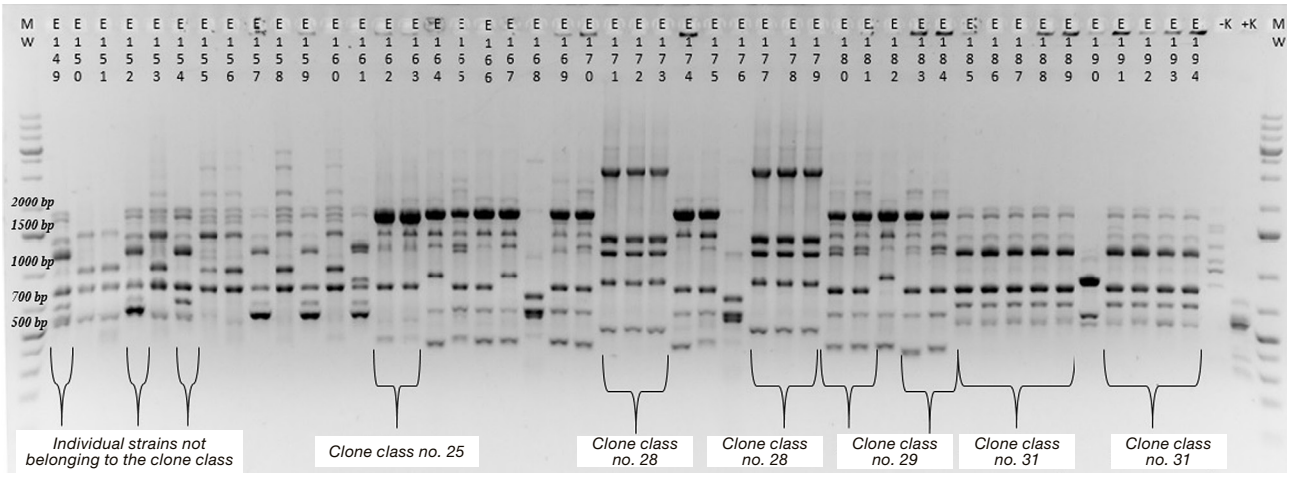

Figure 3. Gel photograph of several clone classes and individual strains (samples: E149–E194, positive control: E31, negative control: distilled water, molecular marker: WM; Gene Ruler 1 kb Plus DNA Ladder)

In the course of our studies, 57 individual strains were found, which could not be classified into clone classes, so the range of isolates was narrowed down to 91 based on the results of the clonality tests. Gram-negative and catalase-positive isolates were excluded by classical microbiological methods, leaving a total of 34 clone classes and 37 individual strains that could not be classified into clone classes, reducing the starting number to 71 isolates. This greatly aided preselection, as less than one third (32.7%) of the strains remained. As the results of the RAPD-PCR assays are highly dependent on laboratory conditions, precise execution of the method is of paramount importance for the reproducibility of the results **[11]**. The 1254 primer used allowed the comparison of isolates with similar patterns in the course of the RAPD-PCR assays. This is consistent with the statement of Torriani et al. **[12]** that primer 1254 is eminently suitable for detecting polymorphisms among L. delbrueckii strains.

4.3. Examination of autoaggregation

In our further studies, the 37 individual strains that could not be classified into clone classes were not included, so the in vitro test series were continued by selecting one bacterial strain from each of the 34 clone classes for the examination of autoaggregation. Our goal was to find well-aggregating (>70% after 5 hours of incubation, >80% after 24 hours of incubation) and non-aggregating (<25%) strains, which then could be included in further acid and bile acid tolerance experiments. It was hypothesized that well-aggregating strains would be more likely to be probiotic, and thus they may also be able to better tolerate acid and bile acid treatment.

Aggregation assay measurements were carried out after 0, 5 and 24 hours. It was decided to perform measurements after 5 hours on the basis of the results of Kos et al. **[13]**, who found that *L. acidophilus M92* was already highly autoaggregated after 5 hours. The authors cultured their test strains in MRS broth to preserve some of the cell surface proteins that allow aggregation **[13]**.

The 34 strains were tested in two biological duplicates. Isolates with an aggregation value over 70% were found after 5 hours of treatment, namely the following six E15, E66, E92, E173, E198 and E216. *L. acidophilus LA-5* and *ATCC 4356* strains used as positive controls also aggregated well (78.2% and 72.1%, respectively) (***Figure 4***).

It should be mentioned that the well-aggregating strains formed pellets visible to the naked eye at the bottom of the Wassermann tubes, and the upper part of the suspension became clear. The same finding was made by García-Cayuela et al. **[9]**, who isolated 126 *L. plantarum* strains from cheese samples made from raw milk and carried out preliminary evaluation of the aggregation (sedimentation) ability of the strains in MRS broth with the naked eye, on the basis of which the appearance of snowflake-like aggregates has been reported. Fourteen strains were included in the autoaggregation study, and optical density measurements were performed after 2, 6, 20 and 24 hours. The highest autoaggregation values (28.5-59.5%) were observed after 1 day. Values increased over time, however, they varied from strain to strain. Compared to the aggregation percentages reported by them, we measured higher values (>75%) after 5 hours of incubation.

Xu et al. **[14]** tested the ability of probiotic and pathogenic strains to self-aggregate. The results obtained after 2 hours of incubation showed that three strains (*Bifidobacterium longum B6, L. rhamnosus GG* and *L. brevis KACC 10553*) performed well, with aggregation percentages between 40 and 50%. Tuo et al. **[15]** examined the aggregation ability of 22 *Lactobacillus* strains after 5 hours of incubation at 37 °C, and values of 24.2 to 41.4% were obtained. They used *L. rhamnosus GG* as a positive control, which proved to be the best performing strain with an aggregation value of 41.4%.

Cumulative results of the autoaggregation study of Transylvanian and control strains (after 5 and 24 hours of incubation) are shown in ***Figure 5***. It can be stated that each strain achieved a higher value after 24 hours compared to its result after 5 hours. The probiotic *L. acidophilus LA-5* used as a control and *L. acidophilus ATCC 4356*, which has a well-aggregating phenotype, performed excellently after 24 hours, as reported in the literature (94.1% and 93.5%, respectively). Of the strains belonging to the 34 clone classes, 19 aggregated above 80%. This means that the method developed by us proved to be suitable to distinguish between well and poorly aggregating isolates. In a 24-hour autoaggregation study of *Lactobacillus* strains isolated from yogurts, Prabhurajeshwar and Chandrakanth **[16]** measured values that were lower than our results (39.4-52.0%).

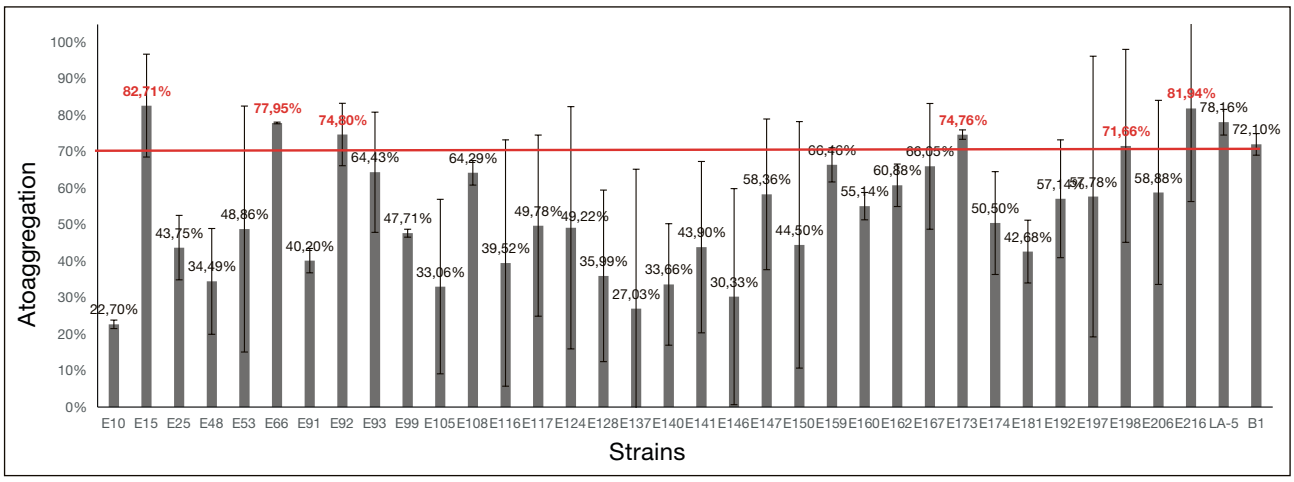

Figure 4. Results of autoaggregation studies of our own isolates and control strains after 5 hours of incubation [Data represent mean ± standard deviation of 2 biological x 3 technical replicates; the horizontal red line allows the visualization of well-aggregating (>70%) strains]

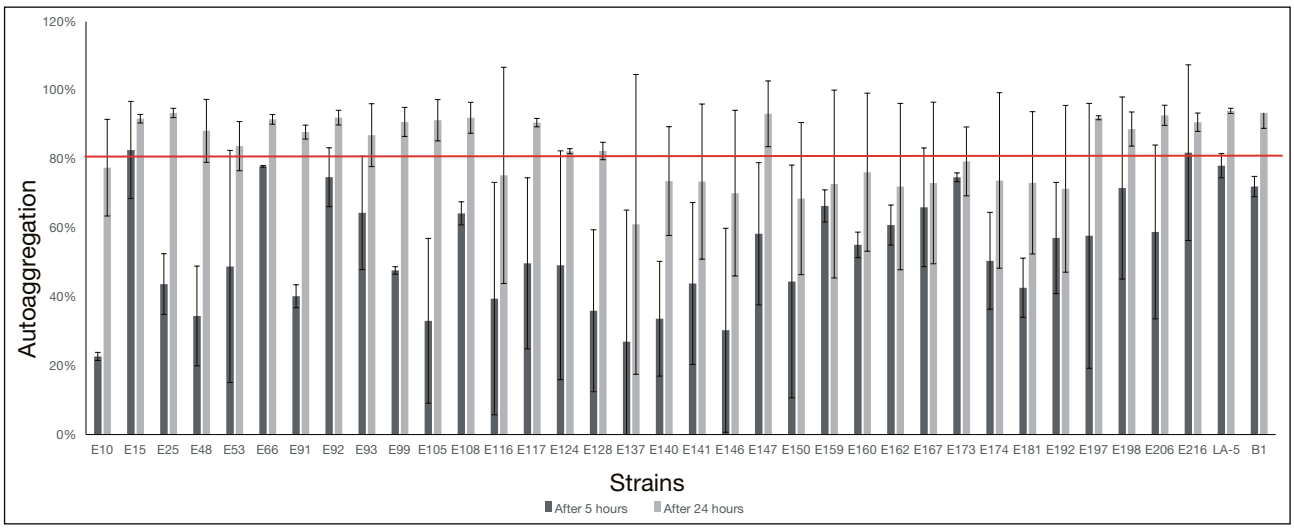

Figure 5. Results of autoaggregation studies of our own isolates and control strains after 5 and 24 hours of incubation [Data represent mean ± standard deviation of 2 biological x 3 technical replicates; the horizontal red line allows the visualization of well-aggregating (>80%) strains]

4.4. Examination of acid and bile acid tolerance

Acid and bile acid tolerance was studied using 6 strains (E15, E66, E92, E173, E198, E216) that aggregated well after 5 hours of incubation, and *L. acidophilus LA-5* was used as a positive control, the latter strain being probiotic, having adequate aggregation indices and having displayed excellent properties in similar studies in the past **[17]**. In addition, from our own isolates, strain E10 with less favorable aggregation ability (22.7%) was also included in our studies in order to determine whether there is a correlation between good aggregation and between acid and bile acid tolerance.

Results were recorded after 48 hours of incubation. At the site of the bacterial suspension droplets with a volume of 10 μL inoculated onto the surface of the culture medium, colony growth or the absence of proliferation was observed. It was judged with the naked eye whether the strains were able to visibly proliferate on the surface of the culture media supplemented with acid or bile acid, as well as on the surface of the control culture media (presence-absence test). On the one hand, we were looking to answer the question what acid and bile acid concentrations the individual strains were able to tolerate and, on the other hand, whether there is a correlation between the aggregating ability and the acid or bile acid tolerance. Our results are shown in ***Tables 4*** and ***5***.

*Table 4. Results of the presence-absence test performed on the surface of solid culture medium supplemented with acid**

Strain	PH value of MRS agar				
	6.0	5.5	5.0	4.0	3.0
E10 isolate	+++	++	++	++	0
E15 isolate	+++	+++	+++	+++	0
E66 isolate	+++	+++	+++	+++	0
E92 isolate	+++	+++	+++	+++	0
E173 isolate	+++	+++	+++	+++	0
E198 isolate	+++	+++	+++	+++	0
E216 isolate	+++	+++	+++	+++	0
Lactobacillus acidophilus LA-5	+++	+++	+++	++	0

* n = 18 (9 parallels × 2 replicates).
0: no proliferation, +: poor proliferation, ++: moderate proliferation, +++: clearly visible, strong proliferation.

*Table 5. Results of the presence-absence test performed on the surface of solid culture medium supplemented with bile acid**

Strain	MRS agar supplemented with			
	0%	0.1%	0.2%	0.5%
	porcine bile			
E10 isolate	+++	+	0	0
E15 isolate	+++	+++	+++	+++
E66 isolate	+++	+++	+++	+++
E92 isolate	+++	+++	+++	+++
E173 isolate	+++	+++	+++	+++
E198 isolate	+++	+++	+++	+++
E216 isolate	+++	++	++	++
Lactobacillus acidophilus LA-5	+++	+++	++	+

* n = 18 (9 parallels × 2 replicates).
0: no proliferation, +: poor proliferation, ++: moderate proliferation, +++: clearly visible, strong proliferation.

It can be seen that *L. acidophilus LA-5* grew well on MRS culture media with pH values of 6.0, 5.5, 5.0 and 4.0, as well as on MRS culture media containing 0.1% and 0.2% bile acid, thus it proved to be well tolerant of acid and moderately tolerant of bile acid. The control strain showed only a week growth on culture media containing 0.5% bile acid. Neither the control, nor the Transylvanian strains formed colonies on the most acidic (pH = 3.0) MRS culture medium, so solid culture media with pH values of 4.0 and 3.0 proved to be suitable for pre-selection.

Pan et al. **[18]** maintained a *L. acidophilus NIT* strain isolated from infant feces in a glycine–hydrochloric acid buffer (pH: 2.0; 3.0; 4.0) for 1, 2 or 3 hours. After the treatment, the bacterial pellet was resuspended, and 20 µL of the suspension of the appropriate dilution members was spread on the surface of MRS agar plates. It was found that after 3 hours of treatment, only 10% of *L. acidophilus* cells survived. Although our studies were not performed in the same experimental setup, the results may explain why *L. acidophilus* did not form colonies on a culture medium with a pH value of 3.0. By the addition of 3% whey protein isolate, Vargas et al. **[19]** achieved that *Streptococcus thermophilus ST-M5* and *L. delbrueckii subsp. bulgaricus LB-12* survived acid treatment in maximum numbers. The aim of Valente et al. **[20]** was to assess the in vitro and in vivo probiotic potential of lactic acid bacterial strains (*L. plantarum B7* and *L. rhamnosus D1*) isolated from traditional Brazilian cheeses. Both strains were moderately tolerant of 0.3% of ox bile after 12 hours of incubation. Both isolates B7 and D1 have been shown to be resistant to artificial digestive juices (pH: 2.0 and 3 g/L pepsin) **[20]**.

The physiological concentration of bile acid salts varies between 0.3% and 0.5% in the gastrointestinal tract **[21]**, this is why 0.5% was chosen as the highest bile concentration.

The author mentioned also added to his culture medium 0.3, 0.5, 1.0 or 2.0% bile acid salt, and then 10 µL of the stock culture was applied to the surface of the culture medium. Although he worked not with Lactobacillus, but with Lactococcus strains isolated from raw cow's and goat's milk and from traditional kefir, his experimental system was similar to ours. Lactococcus lactis strains did not tolerate any of the bile acid concentrations used.

Based on our results, it was found that the selected isolates generally well tolerated the presence of 0.5% bile acid, which in turn was not true for strain E10, which barely proliferated even at the lowest (0.1%) bile concentration. The poor aggregation ability of isolate E10 was accompanied by good acid tolerance and poor bile acid tolerance. The control strain *L. acidophilus LA-5*, although poorly, but still proliferated on the culture medium containing 0.5% bile. During the procedure used, the strains were exposed to the destructive ingredients not only for a few hours, but they were in contact with them for 48 hours. It is worth mentioning that the negative effects of the destructive agents can be mitigated by the addition of whey protein powder to the culture medium **[19]**. Presence-absence testing on the surface of the solid culture medium supplemented with acid or bile acid can be considered a relatively fast method, because the required culture media can be prepared easily, dropping onto the surface of the culture medium can be performed quickly, so the results are available in a short time.

5. Conclusions

Our efforts to develop some elements of an in vitro test system for the selection of probiotic bacterial strains have proven to be successful. The steps presented here do not necessarily need to be further refined, because they are already capable of the pre-selection of large sets of isolates. Although primer 1254 has been shown to be a good choice, it will be worth performing the RAPD-PCR reaction with other primers in subsequent clonality assays. There was a positive correlation between the results of the aggregation studies and those of the acid and bile acid tolerance tests, however, to factually establish the probiotic properties of the isolated strains, further in vitro studies and in vivo animal experiments are needed. In order to have an even more efficient selection than at present, it seems worthwhile to supplement the test system with other elements, e.g., antibiotic resistance or antimicrobial activity assays.

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