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EVALUATION OF ADHESIVE AND AGGREGATION PROPERTIES OF LACTIC ACID BACTERIA ISOLATED FROM DIFFERENT BIOTOPES

Viktoria Marinova-Yordanova[1], Yoana Kizheva[1],
Iliyana Rasheva[1, 2], Petya Hristova[1]

[1]*Department of General and Industrial Microbiology*
Faculty of Biology, Sofia University "St. Kliment Ohridski"
8 Dragan Tsankov Blvd., Sofia 1164, Bulgaria, viktorijjm@uni-sofia.bg (V.M.Y.);
joana_k@biofac.uni-sofia.bg (Y.K.); pkabad@biofac.uni-sofia.bg (P.H.)
[2]*Centre of Competence "Sustainable Utilization of Bio Resources*
and Waste of Medicinal and Aromatic Plants for Innovative Bioactive Products"
8 Dragan Tsankov Blvd., Sofia 1164, Bulgaria, i_rasheva@biofac.uni-sofia.bg (I.R.)

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ABSTRACT

The objective of this study were fifty strains of seven lactic acid bacteria (LAB) species (L. paracasei, L. casei, L. rhamnosus, L. plantarum, L. fermentum, L. delbrueckii subsp. bulgaricus and L. koreensis), previously isolated from various biotopes (human breast milk, newborn feces, and fermented vegetables). As part of their probiotic potential, their ability for adhesion, biofilm / anti-biofilm formation, auto-aggregation and co-aggregation with different pathogenic species were evaluated.

Based on their capacity to form biofilm, strains were grouped as poor (34 %), moderate (33 %), and strong (33 %) biofilm forming bacteria (BFB). L. fermentum 18V, showed the strongest biofilm formation ability and 70 % anti-biofilm activity against P. aeruginosa. The auto-aggregation capability of LAB ranged from 12 % to 68 %. The highest auto-aggregation (85 %) was established for L. fermentum 12V and L. casei 8V (78 %). The best co-aggregation ability with all tested pathogenic species was demonstrate by L. casei 8V. The highest percentage (85 %) of co-aggregation was determined for L. paracasei strain 29V with L. innocua. The housekeeping genes (ef-Tu, eno, gap, groEl, and srtA) involved in binding mechanisms shown 100 % presence in all of the tested strains. The great ability to adhere to mucin was determined for L. koreensis 35V (1. 9 x 10^6 CFU mL^{-1}). The degree of adhesion varies both between individual species and between strains belonging to the same species. The obtained results showed that the LAB possess strain- and species-specifically probiotic properties with potential applications in the food industry.

Keywords: lactic acid bacteria, biofilm formation, adherence, auto-aggregation/co-aggregation properties, cell-surface related molecules, genetic determinants.

INTRODUCTION

The potential health benefits of lactic acid bacteria (LAB) include enhancing the function of the epithelial barrier and preventing pathogen invasion [1]. Multiple mechanisms of action for the beneficial effect of probiotics have been proposed [2, 3]. Potential probiotics are chosen primarily based on the adherence and colonization of lactic acid bacteria strains in the gastrointestinal tract (GIT), which can boost the bacteria's persistence in the gut and provide them an advantage over other species in this environment [4, 5]. Attachment of probiotic bacteria to intestinal cells can have long-term health benefits, including pathogen exclusion, immunomodulation, and the synthesis of helpful bacterial compounds [6]. Binding is a crucial function of LAB and is often of a primary focus of research on their probiotic properties. Over the past decade, studies on the protein molecules related to adhesion have led to a better understanding and have been classified into five classes: anchorless housekeeping proteins, surface layer proteins, LPXTG-motif proteins, transporter proteins, and ‘other’ proteins [7].

Probiotic bacteria first bind to mucin before it reaches the epithelial layer of the intestine. Intestinal pathogens possess enzymes that degrade mucin and cross the protective barrier, leading to infection [8]. Therefore, examining how lactobacilli strains adhere to immobilized mucin offers a valuable knowledge about their competitive inhibition with enteropathogens. However, the mechanism by which probiotic strains interact with mucin components is still poorly understood. The characterization of the factors involved in the surface adhesion of different lactobacilli could allow an understanding of the adhesion mechanism [9].

It’s interesting to note that despite missing traditional secretion signals, several cytoplasmic “housekeeping” proteins have been found in the extracellular proteome. Depending on their location, these proteins - often referred to as “moonlighting proteins” - display a variety of functions, such as immunological regulation and adhesion [10]. Several probiotic strains have been found to contain significant adhesins, including proteins like enolase (ENO), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and elongation factor Tu (EF-Tu) [11]. Twenty-nine surface-associated proteins were found in a thorough proteome analysis of *Lactiplantibacillus plantarum* 299v by Beck et al. [12]. Glycolysis-related enzymes, elements of the transcriptional and translational machinery (such as ribosomal proteins, EF-Tu, and EF-TS), stress response and folding proteins (DnaK, GrpE, GroEL, and GroES), and proteins connected to cell envelope production were among them. Numerous of these proteins shown affinity for host-derived substances such fibronectin, plasminogen, and mucin, corroborating their hypothesized functions in mucosal colonization and host contact [12].

The production of biofilms by certain species of LAB has been documented, and several investigations have identified genes associated with adhesion or biofilm formation. According to Frola et al. the production of a beneficial biofilm by colonizing the inner surfaces of the udder and so forming a barrier against pathogenic microbes is an important element for the probiotic potential of lactic acid bacteria [13]. Furthermore, the adhesion capacity to the epithelium provides for the ability of bacterial strains to continue their antimicrobial activities over time by maintaining their presence in the host [14, 15]. These effects can be achieved by the secretion of inhibitory molecules such as bacteriocins and other antimicrobial chemicals [15]. It is generally accepted that one of the major advantages of probiotics is that their interaction with the epithelium may also cause immunological responses in the lamina propria [14]. Two phases are involved in establishing the close interaction between bacteria and epithelium that is required for the probiotic characteristics listed above: unspecific binding by physicochemical contact; irreversible bacterial attachment, caused by receptor-ligand interactions [16].

Aggregation also serves as the foundation for various physiological functions of probiotics, including resistance to pathogen invasion [17], maintenance of microbial balance within the gut, and involvement in immune regulation [18]. Given that LAB are among the most used probiotic species, comprehensively summarizing their aggregation properties, including both self-aggregation and co-aggregation, is essential. This knowledge provides a foundation for the screening of potential probiotic strains and for analysing their functional attributes.

Lactic acid bacteria inhabit different ecological niches and have evolved various adaptation mechanisms to survive in them. Human breast milk may be considered a natural functional food since it has been shown to have a distinct and intricate food matrix [9]. Traditionally, lactobacilli and *Bifidobacterium* species are most often isolated from breast milk, and some of their representatives (*Lactobacillus gasseri*, *Ligilactobacillus salivarius*, *L. rhamnosus*, *L. plantarum*, *L. fermentum*, *Limosilactobacillus reuteri*, *Bifidobacterium breve*, *Bifidobacterium longum* and others) have GRAS (Generally Recognized as Safe) and QPS (Qualified Presumption of Safety) status and have aroused strong interest in their use as probiotic bacteria [19].

Isolation of LAB from infant feces may also be a suitable niche for potential selection of probiotics, as these strains are resistant to low pH, gastric juice, and bile salts [20]. Their adaptation to two different environments, the extraintestinal environment such as food and the human intestine (in terms of colonization and persistence), exerting different selective pressures related to their growth rate and carbohydrate metabolism, gives reason to consider them as probiotics [21, 22].

For centuries, fermented products made from plant and animal materials have been a vital component of the human diet across various regions of the world. The study of the beneficial microbial diversity in these naturally fermented foods is important, especially for the selection, isolation, and biotechnological use of potential probiotic bacterial strains [23]. In fermented vegetable products, *L. plantarum*, *Levilactobacillus brevis*, and *Leuconostoc mesenteroides* are usually the predominant microbiota [24].

The primary objective of this study is to evaluate the phenotypic and genotypic characterization of adhesion properties of members of the *Lactobacillaceae* family isolated from three distinct biotopes (breast milk, newborn feces, and fermented vegetables). Several key probiotic abilities of the tested isolates were determined in vitro by investigation of the degree of adhesion, biofilm and antibiofilm formation, and ability to co-aggregate to pathogenic species.

EXPERIMENTAL

Bacterial strains and growth conditions

Fifty LAB strains isolated from breast milk, infant feces, fermented vegetables, used in this study, were taken from the culture collection of the Department of General and Industrial Microbiology, Faculty of Biology, Sofia University "St. Kliment Ohridski". Generally, seven LAB species were used: *Lacticaseibacillus paracasei* (n = 15), *Limosilactobacillus fermentum* (n = 11), *Lacticaseibacillus casei* (n = 9), *Lacticaseibacillus rhamnosus* (n = 9), *Lactobacillus delbrueckii* subsp. *bulgaricus* (n = 1), *Lactiplantibacillus plantarum* (n = 4) and *Levilactobacillus koreensis* (n = 1). All used strains were previously identified and characterized [25, 26]. *Pseudomonas aeruginosa* ATCC 3732, *Candida albicans* ATCC 10231, *Salmonella enterica* subsp. *enterica* ser. Enteritidis ATCC 13076, *Escherichia coli* ATCC 25922, *Listeria innocua* strain SLCC 3379 ATCC 33090 and *Staphylococcus aureus* ATCC 6538 were used as test pathogens. All lactic acid bacteria were stored at - 80^{o}C in de Mann Rogosa Sharpe (MRS) broth (Merck, Germany) with 30 % glycerol. Test pathogens were maintained on either Brain Heart Infusion agar (BHI, Merck KGaA, Darmstadt, Germany) or yeast Peptone Dextrose agar (YPD, Merck KGaA, Darmstadt, Germany) and stored at 4^{o}C. Before experimental use, cultures were subculture twice in MRS, BHI or YPD at 37^{o}C for 24 h.

Biofilm formation assay

The LAB isolates were evaluated for their potential to form biofilm using the crystal violet method [27]. A 100 μL of overnight LAB cultures (approximately 10^8 CFU mL^{-1}) were added into the microtiter polystyrene plate wells which were previously filled with 100 μL of MRS broth. The cells were allowed to adhere at 37^{o}C for 24 h. The non-adherent cells were removed by washing the wells three times with 200 μL of Phosphate-buffered saline (PBS, pH 7. 4). The adhered cells were stained with crystal violet (100 μL/well, 0. 1 %, w/v, solution) for 30 min. Wells were then washed five times with PBS to remove the excess stain. The plate was then left to dry out for 30 min, and the absorbance was measured at 640 nm using the SPECTROstar® Nano Microplate Reader (BMG LABTECH, Ortenberg, Germany). The negative control included wells containing non-inoculated broth. Results were calculated by subtracting the absorbance of the negative control from the absorbance value documented for each inoculated well, expressed in percentage. Based on the optical density (OD), biofilm-forming bacterial (BFB) strains were classified as: non-BFB (OD ≤ ODC), weak BFB (ODC < OD ≤ 2 × ODC), moderate (2 × ODC < OD ≤ 4 × ODC) or strong BFB (4 × ODC < OD) according to Borges et al. [28].

Anti-biofilm activity

According to the methods described by Cui et al. [29], the tested LAB strains were evaluated for anti-biofilm activity against the test pathogens *P. aeruginosa* ATCC 3732 and *C. albicans* ATCC 10231. The percentage anti-biofilm activity of each isolate was calculated. A 100 μL of overnight cultures of each pathogenic bacterium (10^8 CFU mL^{-1}) were transferred to 96-well microtiter plates followed by the addition of 100 μL of lactic acid bacterial supernatants adjusted to pH 7. 0 and were incubated for 24 h at 37^{o}C. After the incubation, the medium was discarded, and planktonic cells were removed from each well by gently washing twice with sterile PBS. Further, the biofilms formed were fixed with 200 μL methanol for 10 min, stained with 200 μL 0. 1 % crystal violet for 10 min and rinsed thrice with water gently. Crystal violet attached to the biofilm samples was dissolved with 200 µL of 33 % acetic acid. The absorbance was measured at 590 nm using SPECTROstar® Nano Microplate Reader. The test-pathogen cultures used as a positive control did not include cell free supernatant (CFS). BHI and YPD broth without inoculated test microorganisms serve as negative controls. The percentage of biofilm inhibition was calculated using Eq. (1):

$$\text{Biofilm inhibition (\%)} = (A_{\text{growth control}} - A_{\text{sample}}) / (A_{\text{growth control}}) \times 100 \qquad (1)$$

Test for auto-aggregation and co-aggregation

Auto-aggregation and co-aggregation assays were carried out according to Tuo et al. [30]. The LAB isolates were grown on MRS broth overnight at 37^{o}C. For auto-aggregation, the bacterial cells were harvested by centrifugation at 10 000 x g for 10 min at 4^{o}C, washed twice with PBS (pH - 7.2), and then resuspended in the PBS till obtaining the cell concentration (10^8 CFU mL^{-1}, OD 600 nm = 0.25). The auto-aggregation was measured with SPECTROstar® Nano Microplate Reader at 600 nm (Initial) at three time points: 0 h, 4 h and 24 h. The auto-aggregation percentage was calculated by Eq. (2):

$$\text{Auto-aggregation (\%)} = [1 - A_t / A_0] \times 100 \qquad (2)$$

where A_t represents the absorbance at time t = 4 h or 24 h and A_0 the absorbance at t = 0.

The co-aggregation (CA) was carried out with mixtures of equal volumes (5 mL) of LAB strains and pathogenic test bacteria. Standardized suspensions, prepared in PBS as described above (10^8 CFU mL^{-1}), were obtained for all used microorganisms: LAB, *S*. Enteritidis, *E. coli*, *L. innocua* and *S. aureus*. The resulted mixtures were incubated at 37^{o}C without agitation for 24h. The co-aggregation percentage was measured at 600 nm (A 600nm) at three time points: 0 h, 4 h and 24 h and calculated according to Eq. (3):

$$\text{Co-aggregation (\%)} = [(A_0 - A_t) / A_0] \times 100 \qquad (3)$$

where A_0 represents the absorbance of the mix immediately after mixing and A_t represents the absorbance of the mix at time 4 h or 24 h [31].

Detection of binding-related genes by PCR amplification

The total DNA of all strains was isolated from overnight MRS broth cultures using a Tissue and Bacterial DNA Purification kit (EURx Ltd., Poland) according to manufacturer's instructions. The extracted DNA was purified using the PCR / DNA Clean-up Kit (GeneMatrix, EURx Ltd., Gdansk, Poland) and stored at - 20^{o}C. The genes involved in binding

mechanisms (*slpA*, *mub1*, *mub2*, *msa*, *mapA*, *gtf*, *fpbA*, *cnb*, *apf*, *cbsA*) and housekeeping genes (*ef-Tu*, *eno*, *gap*, *groEl*, *srtA*) were examined by PCR with specific primers according to Turpin et. al. [32]. Each PCR reaction mixture was prepared in a final volume of 25 µL with the following composition: Red Taq polymerase master mix – 6. 5 µL; 0. 5 µL of each primer; sterile water - 16. 5 µL; DNA - 1 µL. The PCR conditions were: initial denaturation at 95^{o}C for 5 min, followed by 40 cycles (95^{o}C for 30 s, the appropriate annealing temperature depending on the primer used for 10 s, and 72^{o}C for 15 s), followed by final elongation at 72^{o}C for 5 min. Amplification products were visualized electrophoretically on a 1. 5 % agarose gel for 30 min. at 100 V. A 100 bp DNA Ladder (Fisher Scientific International Inc.) was used. The gel was stained in ethidium bromide solution for 15 min, and the fragments were visualized under UV light.

Mucin adhesion assay

Lactobacilli strains were assayed for adhesion to immobilized mucin [33]. The wells of a 96-well microtitre plates (Nunc, Nun-clone Delta SI, Denmark) were coated with 300 µL of purified porcine mucin (0. 5 mg mL^{-1} Sigma-Aldrich) in sterile PBS under sterile conditions and incubated overnight at 4^{o}C at the fridge. Unbound mucin in each well was removed through washing with sterile PBS twice. Controls consisted of PBS-treated wells and untreated wells. The results of at least four re-plicates were used to estimate the adhesion ability of a tested strain. The overnight grown bacterial cells were harvested by centrifugation (10 000 g for 2 min, at 4^{o}C) and washed twice with sterile PBS. The cell density was adjusted to 10^8 CFU mL^{-1} in sterile PBS (OD 600 nm - 0. 25) and the number of bacteria was parallel determined via the plate count method on MRS agar. Two hundred microliter (200 µL) of each strain was added to respective wells containing immobilized mucin and allowed to adhere for 90 min at

37°C. Un-adhered bacterial cells were then withdrawn, and wells were washed five times with 300 μL sterile PBS to remove unbound bacteria. Bacteria bound to mucin were released using 300 μL 0. 05 % (v / v) Triton X-100 in sterile PBS for 20 min at 37°C. The number of bacteria that adhere to mucin was determined using the plate count method on MRS agar after appropriate dilution, and enumeration was carried out following 48 h incubation at 37°C. Prior to use, Triton X-100 concentration was tested to determine the influence of the reagent on bacterial viability. It was also determined that 30 min treatment of 0. 05 % (v / v) Triton X-100 in sterile PBS at 37°C did not affect the viability of lactobacilli.

Data analysis

All experiments were performed in triplicate. The obtained data were analysed by Microsoft Excel built in functions, and the results were expressed as mean ± standard deviation.

RESULTS AND DISCUSSION

Biofilm formation

Bacterial biofilms are important key to understanding how bacteria adapt to environmental stress and colonize various niches. Lactic acid bacteria biofilms provide a barrier that prevents harmful microbes from adhering to mucosal surfaces [34]. Furthermore, exopolysaccharides, which are secreted by BFB, provide customers with health benefits by either improving the sensory qualities of food or acting as indigestible fiber [35].

The capacity to form biofilms was demonstrated for all LAB strains used in this study, to various degrees (Fig. 1). Accordingly, the strains were categorized as follows: weak BFB - 34 % of the strains (1V, 7V, 8V, 9V, 13V, 19V, 26V, 36V, 40V, 37V, 38V, 41V, 42V, 49V, 43V, 50V, 47V); moderate BFB - 33 % (2V, 5V, 10V, 12V, 14V, 15V, 20V, 21V, 22V, 29V, 30V, 31V, 32V, 34V, 45V, 48V); strong BFB - 33 % of the strains (3V, 4V, 6V, 11V, 16V, 17V, 18V, 23V, 24V, 25V, 27V, 28V, 33V, 39V, 46V, 44V). Notably, four strains (6V, 23V, 25V, and 44V) produced more than 80 % of the biofilm. The strain *L. fermentum* 18V appeared as the strongest BFB, with 100 % productivity. Significant variations between strains of the same species are revealed by the biofilm formation assay results (Fig. 1). Among the eleven strains of *L. fermentum* production of biofilm varies from 40 % to 100 %. Also, three strains of *L. paracasei* showed different ability to form biofilm: strain 13V (17 %) was a weak BFB, strain 2V (36 %) was a moderate BFB and strain 16V (75 %) was a strong BFB. These data clearly indicate that

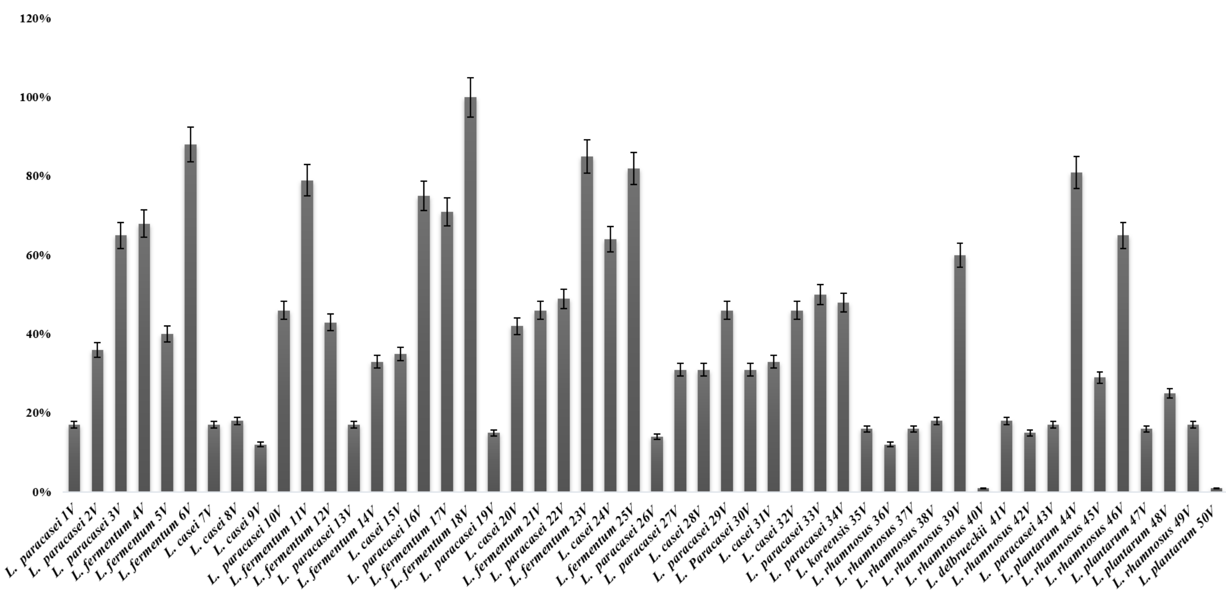

Fig. 1. Ability of LAB strains to form biofilm.

the ability to form biofilm is strain-specific. Our results are in accordance with those reported from Kubota et al. for strains of *L. plantarum* and *Levilactobacillus brevis* [36]. In another study, the biofilm formation ability among four *L. fermentum* strains has been shown to vary between 36 % and 83 %. This suggests that biofilm formation could potentially be influenced by environmental factors and the microbial composition of the ecological niche according to Atanasov et al. [37]. A study by Gómez et al. reported strain-dependent biofilm formation in *Lactococcus lactis* and *Lactobacillus curvatus* strains [38]. Similarly, strain-specific biofilm formation ability (19 % to 86 %) has been reported for six examined LAB strains, according to Jha et al. [39].

Anti-biofilm activity

The cell-free supernatants (CFS) of various LAB strains, containing bacteriocins, organic acids, biosurfactants, hydrogen peroxide, reuterin and diacetyl have been widely used both to inhibit and control the growth of pathogenic bacteria and to disrupt their associated biofilms [40].

The ability of all our LAB isolates to produce anti-biofilm formation substances against *P. aeruginosa* and *C. albicans*, was investigated. The CFS of five *L. fermentum* strains (4V, 11V, 12V, 18V, 21V,) and two *L. paracasei* strains (16V, 33V) effectively inhibited the biofilm of *P. aeruginosa* more than 50 %. The *L. fermentum* 18V strain produced substances that inhibit up to 70 % the ability of the pathogen to form biofilm. The remaining isolates showed a very low percentage or complete lack of ability to inhibit *Pseudomonas* biofilm formation (Fig. 2).

A number of authors have reported that some LAB strains can inhibit biofilms formed by both Gram-positive and Gram-negative bacteria, although these

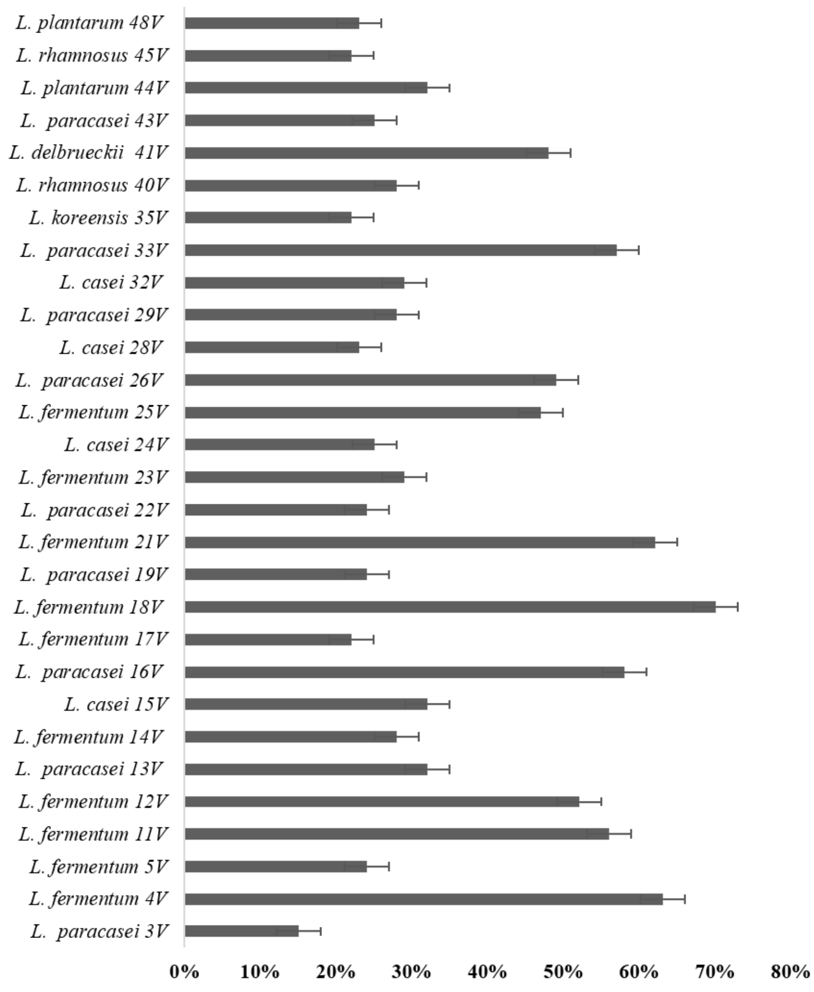

Fig. 2 Anti-biofilm formation against *P. aeruginosa* ATCC 3732.

abilities depend on the strain [30]. Hoseinian et al. have reported that strains *L. casei* and *L. plantarum*, isolated from milk and yogurt, could inhibit (11 % and 13 %, respectively) the biofilm formation of *P. aeruginosa* [41]. In milk environment, *L. plantarum* has demonstrated the greatest inhibition of pathogenic biofilm formation (26 %), while *L. casei* - 16 %. Our results are consistent with two Argentinian studies where *L. casei*, *L. acidophilus*, and *L. plantarum* strains have been reported to inhibit the biofilm production ability of *P. aeruginosa* [42].

A part of the studied lactic acid bacteria showed anti-biofilm ability also against *C. albicans* (Fig. 3). Only five strains - 3V, 10V, 20V, 36V and 43V - produced substances that inhibited biofilm formation by more than 50 %. Among them, the *L. casei* 20 V strain (69 %) showed the highest activity against *C. albicans* biofilm. The remaining isolates showed either a very low percentage or a complete lack of anti-biofilm activity against this pathogen. The analyses performed show that the ability to form substances that have antibiofilm activity is again a strain-specific property. In a similar study, the cell-free filtrates of *L. acidophilus* 8MR7 and *L. paracasei* subsp. *paracasei* 10MR8 inhibited the formation of biofilms of the *Candida* spp. [43]. In addition, the organic acids such as lactic acid and hydrogen peroxide secreted by lactic acid bacteria inhibit the growth of *C. albicans*. Gudiña et al. reported that the ability of *L. acidophilus* and *L. paracasei* subsp. *paracasei* A20 to inhibit the adhesion of *Candida* species was low [44]. Their findings are consistent with our data.

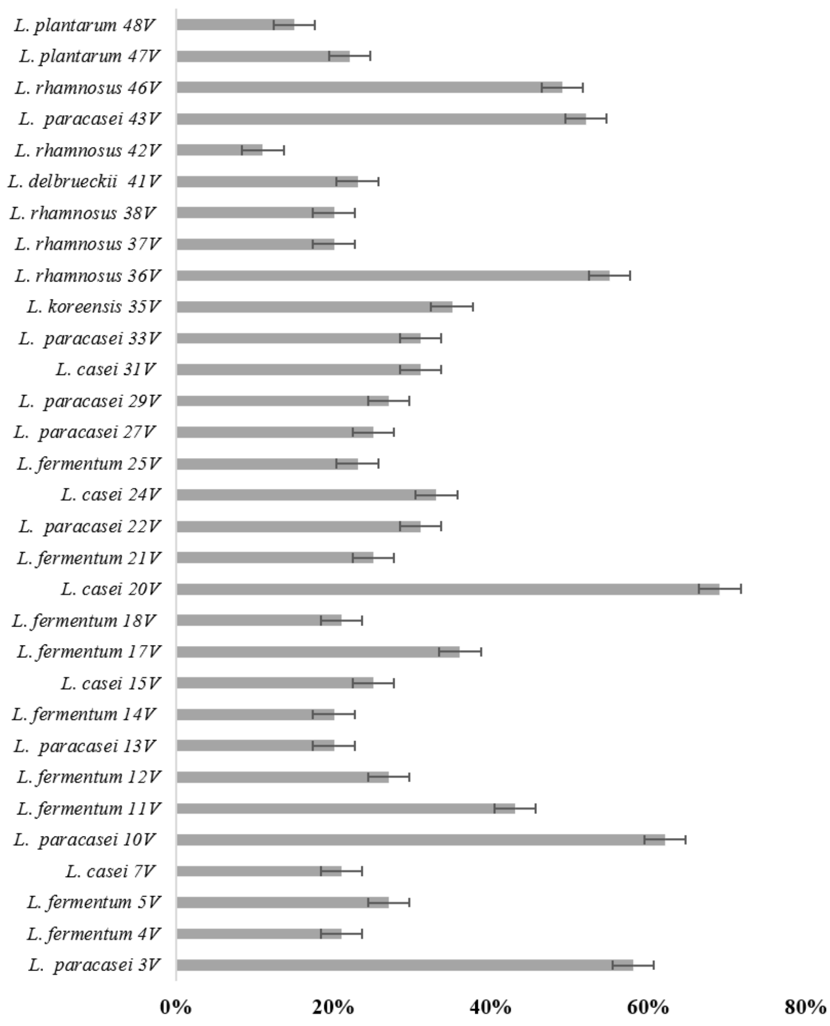

Fig. 3 Anti-biofilm formation against *C. albicans* ATCC 10231.

Auto-aggregation and co-aggregation assay

Auto-aggregation allows the cells of the same strain to form clumps and cling to surfaces, whereas co-aggregation causes intercellular adhesion between distinct microorganisms [39, 45]. The ability to auto-aggregate is an essential property of LAB to form a barrier on GIT mucosa which prevent the attachment of pathogenic bacteria to it. There is still no single scale for determining the degree of auto-aggregation. According to some authors, lactobacilli have an auto-aggregation capacity ranging from low to moderate and 40 % are considered significant [46]. To verify the auto-aggregation potential of our strains, all fifty isolates were tested for their ability to auto-aggregate. The results were analysed at 4 and 24 h of the experiments. Table 1 represent the results for the all-tested LAB strains.

Among the lactobacilli examined, 28 % exhibited a high auto-aggregation ability, greater than 40 %. Generally, the highest percentage of auto-aggregation was observed for two strains: *L. casei* 8V (78 %) and *L. fermentum* 12V (85 %) at 24th hour, and the lowest auto-aggregation ability - for strains *L. paracasei* 2V and *L. rhamnosus* 45V, at 4th h. It turns out that the strains of the *L. fermentum* species show different degrees of auto-aggregation (7 to 85 %). Notably, six strains of *L. casei* exhibited a high level of auto-aggregation (over 40 %). The auto-aggregation capability of the rest LAB ranged from 12 % to 68 %. Auto-aggregation ability is strain-specific, as isolates of the same species have different aggregation potential. In recent years, several authors have investigated the auto-aggregation abilities of lactobacilli, as part of their probiotic potential.

Table 1. Auto-aggregation ability of all tested LAB strains.

Strains	Auto-aggregation, %		Strains	Auto-aggregation, %	
	4 h	24 h		4 h	24 h
L. paracasei 1V	24	31	*L. paracasei* 26V	18	53
L. paracasei 2V	5	7	*L. paracasei* 27V	42	58
L. paracasei 3V	23	50	*L. casei* 28V	37	42
L. fermentum 4V	13	21	*L. paracasei* 29V	28	30
L. fermentum 5V	15	16	*L. paracasei* 30V	46	48
L. fermentum 6V	17	24	*L. casei* 31V	25	26
L. casei 7V	18	30	*L. casei* 32V	44	47
L. casei 8V	41	78	*L. paracasei* 33V	49	56
L. casei 9V	15	19	*L. paracasei* 34V	18	19
L. paracasei 10V	18	29	*L. koreensis* 35V	17	65
L. fermentum 11V	19	22	*L. rhamnosus* 36V	6	8
L. fermentum 12V	78	85	*L. rhamnosus* 37V	15	21
L. paracasei 13V	12	13	*L. rhamnosus* 38V	21	26
L. fermentum 14V	33	68	*L. rhamnosus* 39V	20	26
L. casei 15V	39	60	*L. rhamnosus* 40V	14	16
L. paracasei 16V	16	19	*L. bulgarcus* 41V	7	10
L. fermentum 17V	6	7	*L. rhamnosus* 42V	23	30
L. fermentum 18V	15	15	*L. paracasei* 43V	15	22
L. paracasei 19V	26	27	*L. plantarum* 44V	20	26
L. casei 20V	40	52	*L. rhamnosus* 45V	3	3
L. fermentum 21V	12	14	*L. rhamnosus* 46V	20	27
L. paracasei 22V	26	27	*L. plantarum* 47V	20	26
L. fermentum 23V	30	40	*L. plantarum* 48V	8	17
L. casei 24V	39	45	*L. rhamnosus* 49V	14	27
L. fermentum 25V	16	20	*L. plantarum* 50V	15	23

[*]The strains marked in gray are those that show auto-aggregation ability greater than 40 % at 24th h.

Lactobacilli investigated by Gomaa et al. have exhibited excellent auto-aggregation properties (from 51 % to 78 %) at the 5th hour of incubation [47]. In our isolates experiments, such a high percentage of auto-aggregation was observed after incubation of 24 h. High auto-aggregation was also reported for the strains of *L. casei* and *L. fermentum*, ranging from 61 % to 96 % [48].

As a crucial host defence mechanism against infections in the gastrointestinal and urogenital tracts, co-aggregation between lactobacilli and pathogenic microorganisms helps to form a barrier that stops their adherence to the epithelia and subsequent access to the tissues [47]. Co-aggregation capacity against four types of harmful bacteria was evaluated in this study: *E. coli*, *S*. Enteritidis, *L. innocua* and *S. aureus*.

Like the assessment of auto-aggregation in lactic acid bacteria, there is no universally accepted scale for determining whether a strain exhibits high or low co-aggregation capacity against a given pathogen. Therefore, based on the results obtained in our study on the co-aggregation potential of lactic acid bacterial isolates, we propose a preliminary scale which divide the strains into three categories: weak co-aggregators (co-aggregation with the pathogen up to 20 %), moderate co-aggregators (from 21 % to 40 %), and strong co-aggregators (above 41 %).

Highest co-aggregation between *S*. Enteritidis and *L. casei* 8V (43 %) was observed at 4th h (Fig. 4a). Conversely, eight of the strains (17V, 28V, 30V, 34V, 40V, 42V, 44V and 46V) showed low co-aggregation activity (below 10 %) at 4 h. No significant increase in the co-aggregation between these strains and the pathogen was observed, up to 24 h. The remaining studied strains showed higher co-aggregation ability (from 10 % to 45 %) at 24 h. The highest percentage of co-aggregation (51 %) at 24 h was observed for strain *L. paracasei* 10V. According to the preliminary scale we proposed, 66 % of our LAB were identified as weak co-aggregators with *S. enteritidis*, 30 % as moderate, and only 4 % (strains 8V and 10V) as strong co-aggregators. All tested strains of *L. paracasei*, exhibited strain-specific co-aggregation ability with this pathogen (between 8 and 51 %) at 24th hour. All *L. plantarum* strains show low co-aggregation ability against the pathogen, between 5 - 14 % (at 4 and 24 h). It has been reported that the co-aggregation between LAB and three pathogenic bacteria (*S.* Typhimurium, *S. aureus* and *E. coli*), at 4 h, ranged between 10 and 30 %. In addition, these LAB showed a significant increase in co-aggregation at 24th h [49]. The coaggregation abilities of five lactobacilli strain (*L. plantarum* 83, *L. plantarum* 114 and *L. kefir* (8321, 83, 113)), isolated from kefir grains with *Salmonella*, also have shown to be very variable - even strain-specific [50].

The highest rate of co-aggregation was found between *E. coli* and two of ours' LAB strains: *L. casei* 8V (40 %) at 4 h and *L. paracasei* 19V (58 %) at 24 h, respectively (Fig. 4b). According to the observed results, 14 % of tested strains showed low potential of co-aggregation with *E. coli* (below 10 %) at 24 h. The establishment of different percentages (from lowest result of 6 % - 1V to highest of 58 % - 19V) of co-aggregation of *L. paracasei* strains with *E. coli* indicates that the activity against this pathogen is strain-specific. Sophatha et al. described that the co-aggregation of *Lactobacillus* strains after 24 h of incubation with pathogens such as enterotoxigenic *E. coli*, non-enterotoxigenic *E. coli*, *S. enterica*, varied between 35 % and 66 % [51]. Co-aggregation of different *Lactobacillaceae* strains with *E. coli* O157:H7 after 5 h incubation ranges between 21 % and 32 % [52]. According to some authors, the co-aggregation abilities of LAB strains depend on their auto-aggregation properties and on the duration of incubation [53]. According to our preliminary scale, 44 % of investigated LAB strains were weak co-aggregators with *E. coli*, 48 % were moderate, and 8 % - strong co-aggregators. Notably, strain 8V (*L. casei*) demonstrated a high co-aggregation percentage - exceeding 40 % - with both *E. coli* and *Salmonella*.

The observed tendency of weak co-aggregation capacity of our LAB strains with the two tested Gram-negative pathogens, was also established for Gram-positive test microorganisms (*L. innocua and S. aureus).* Weak co-aggregators with *L. innocua* were 54 %. This group demonstrated low co-aggregation at 4th h, and this activity persisted through 24th h. Six isolates (*L. paracasei* 13V and 30V, *L. rhamnosus* 37V, 42V, and 49V, and *L. plantarum* 50V) had the lowest co-aggregation values, which were less than 10 % (Fig. 4c). Moderate capacity for co-aggregation was observed in 36 % of our LAB strains. Strong co-aggregation activity with *L. innocua* was found only for 10 % of the tested LAB. The highest level was shown by *L. paracasei* strain 29V (85 %) at 24 h. With 79 % co-aggregation with *Listeria* at 24 h, *L. casei* strain 8V once again demonstrated its potent co-aggregation ability, in line with the findings seen with the other two pathogens. The remaining three strong co-aggregators showed ability above 40 % (*L. fermentum* 6V and 21V, and *L. casei* 7V). Some authors have reported that the longer the LAB and pathogenic cells are in touch, the greater the degree of co-aggregation tends to be. The co-aggregation percentage of strain 4 - 10 with *Listeria monocytogenes* remained low during the first 2 h but showed a significant increase over time. This suggests that *L. plantarum* 4 - 10 progressively established interactions with *L. monocytogenes* throughout the co-aggregation process [54].

Evaluating the co-aggregation ability of the tested LAB with the pathogen *S. aureus*, approximately 50 % of all strains were weak co-aggregators. The lowest co-aggregation (1 - 4 %) were observed for three isolates: 36V, 45V, and 50V. Notably, the group of strong co-aggregators included seven isolates (14 %) representing three species: *L. fermentum* (6V, 18V and 23V), *L. paracasei* (19V, 27V), and *L. casei* (7V, 8V). Strong co-aggregation (above 40 %) was observed even at 4 h of incubation for two isolates - 18V and 19V (Fig. 4d). At 24 h of the experiment, *L. paracasei* 19V achieved a co-aggregation rate of 81 % with *S. aureus*. Interestingly, *L. casei* 8V initially exhibited low levels of co-aggregation with *S. aureus* at the 4th h (9 %), but once again it demonstrated its strong aggregation capacity, with the co-aggregation rate increasing to 68 % up to the 24 h.

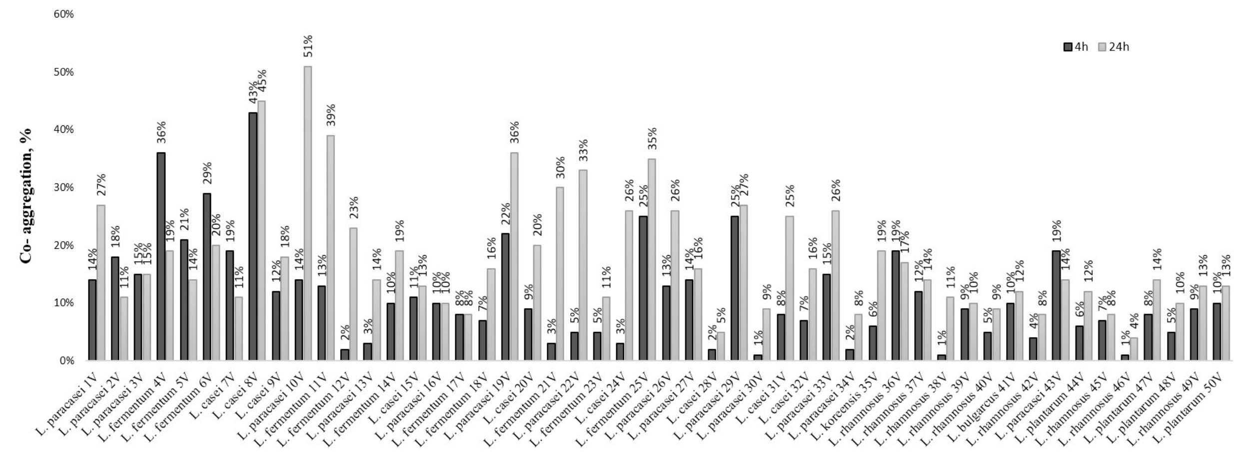

Fig. 4 a. Co-aggregation ability of the tested LAB strains with *S. enterica* subsp. *enterica* ser. Enteritidis ATCC 13076.

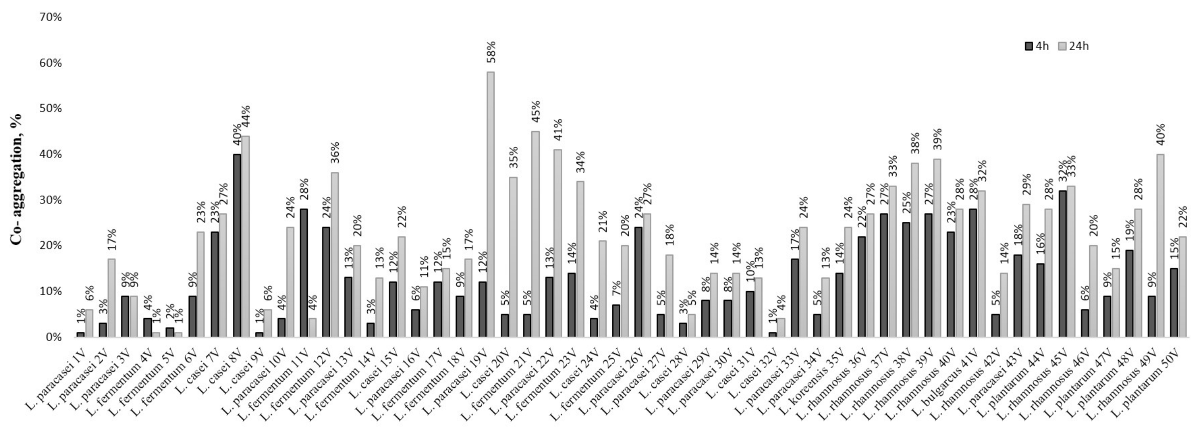

Fig. 4b. Co-aggregation ability of the tested LAB strains with *E. coli* ATCC 25922.

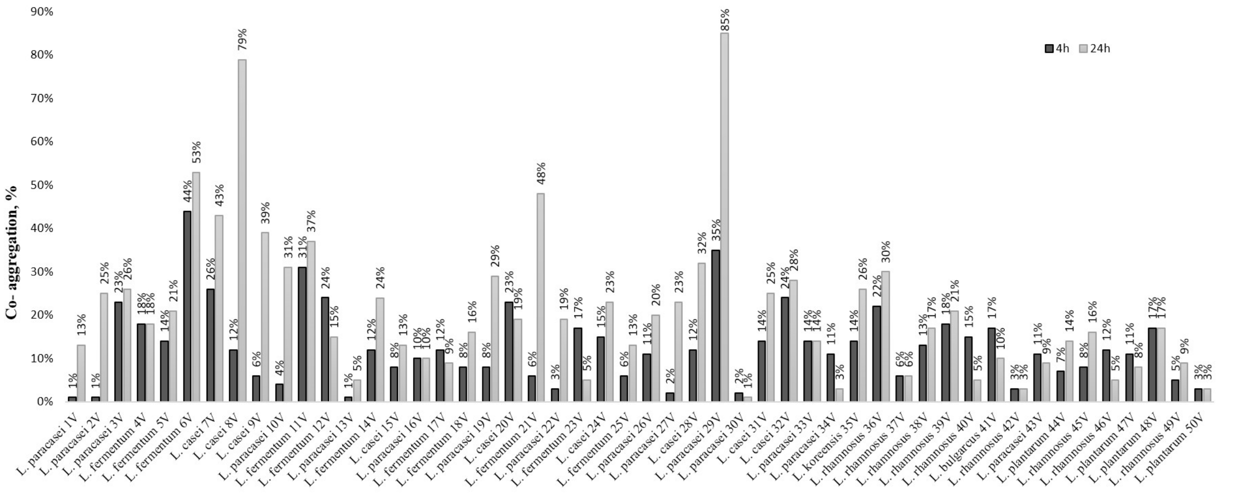

Fig. 4c. Co-aggregation ability of the tested LAB strains with *L. innocua* SLCC 3379 ATCC 33090.

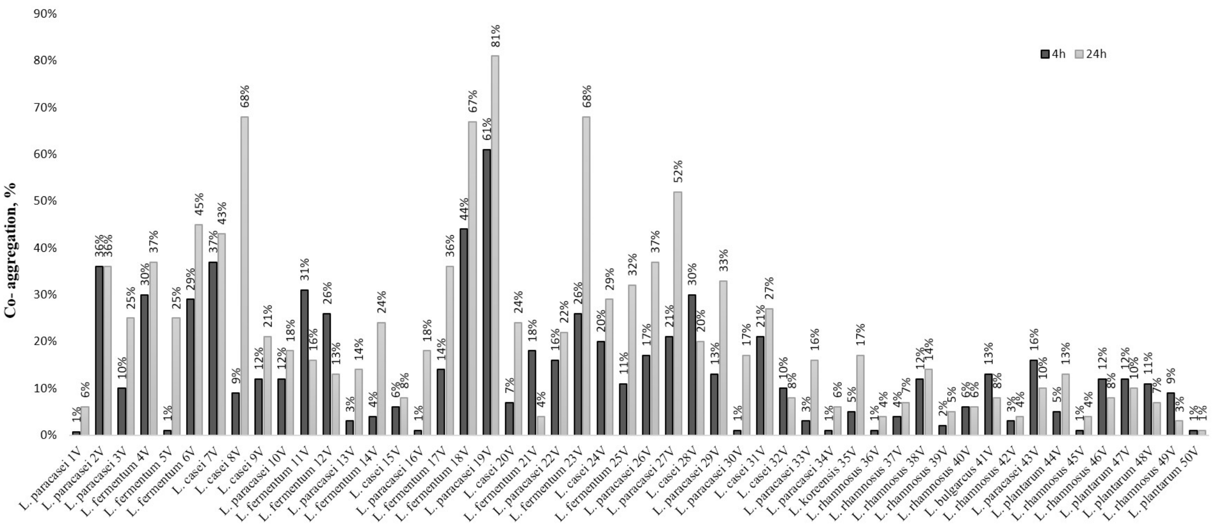

Fig. 4d. Co-aggregation ability of the tested LAB strains with *S. aureus* ATCC 6538.

Detection of genes involved in binding mechanisms

The difference in binding capacity between LAB strains may be explained by variable expression of binding-related genes. At least 20 genes have been reported to play a functional role in *Lactobacillaceae* binding to the digestive tract, with one-third of them recently characterized [55]. In the present study, we screened 14 genes involved in cell binding and analyzed all of our 50 strains to assess their binding ability as a part of their probiotic potential.

The housekeeping genes (*ef-Tu*, *eno*, *gap*, *groEl*, and *srtA*) involved in binding mechanisms shown 100 % presence in all the tested strains. According to Turpin et. al. the same housekeeping genes were present in all their isolates, therefore screening them was not essential to evaluate the bacteria's binding capacity [32]. However, they could serve as positive controls for gene detection.

The genes involved in binding mechanisms (*slpA*, *mub1*, *mub2*, *msa*, *mapA*, *gtf*, *fpbA*, *cnb*, *apf*, *cbsA*) were also examined (Table 2). The *slpA* was not detected in any of our strains. This gene was primarily found in the phylogenetic groups of *L. acidophilus* and *L. brevis*,

Table 2. Distribution of genes involved in binding mechanisms in a collection of LAB strains.

Strain	Binding related genes									
	slpA	*mub*2	*mub*1	*msa*	*mapA*	*gtf*	*fpbA*	*cnb*	*cbsA*	*apf*
L. paracasei 1V	-	-	+	-	-	-	+	+	-	+
L. paracasei 2V	-	-	+	-	-	-	+	+	-	+
L. paracasei 3V	-	-	-	-	-	-	+	+	-	+
L. fermentum 4V	-	-	+	-	-	-	+	+	-	+
L. fermentum 5V	-	-	-	-	-	-	+	+	-	+
L. fermentum 6V	-	+	-	-	+	-	+	+	-	+
L. casei 7V	-	-	-	-	-	-	+	+	-	+
L. casei 8V	-	-	-	-	-	-	+	+	-	+
L. casei 9V	-	-	-	-	-	-	+	+	-	+
L. paracasei 10V	-	+	-	-	+	-	+	+	-	+
L. fermentum 11V	-	+	-	-	-	-	+	+	-	+
L. fermentum 12V	-	+	+	-	-	+	+	+	-	+
L. paracasei 13V	-	+	-	-	+	-	+	+	-	+
L. fermentum 14V	-	+	-	-	-	-	+	+	-	+
L. casei 15V	-	-	-	-	-	-	+	+	-	+
L. paracasei 16V	-	+	-	-	+	-	+	+	-	+
L. fermentum 17V	-	-	-	-	-	-	+	+	-	+
L. fermentum 18V	-	-	+	-	-	-	+	+	+	+
L. paracasei 19V	-	+	-	+	+	-	+	+	-	+
L. casei 20V	-	+	-	-	-	-	+	+	+	+
L. fermentum 21V	-	+	-	-	-	-	+	+	-	+
L. paracasei 22V	-	-	-	-	-	-	+	+	-	+
L. fermentum 23V	-	-	-	-	-	+	+	+	-	+
L. casei 24V	-	+	-	-	+	-	+	+	-	+
L. fermentum 25V	-	+	-	-	+	-	+	+	-	+
L. paracasei 26V	-	+	-	-	-	-	+	+	-	+
L. paracasei 27V	-	+	-	-	+	-	+	+	+	+
L. casei 28V	-	+	-	-	+	-	+	+	-	+
L. paracasei 29V	-	+	-	-	+	-	+	+	-	+
L. paracasei 30V	-	+	+	-	+	-	+	+	-	+
L. casei 31V	-	-	-	-	-	-	+	+	-	+
L. casei 32V	-	+	-	-	-	-	+	+	-	+
L. paracasei 33V	-	+	-	-	-	-	+	+	-	+
L. rhamnosus 34V	-	+	-	-	-	-	+	+	-	+
L. koreensis 35V	-	+	-	+	-	-	+	+	+	+
L. plantarum 36V	-	+	-	-	-	-	+	+	-	+
L. rhamnosus 37V	-	+	-	+	-	-	+	+	+	+
L. rhamnosus 38V	-	+	-	-	-	-	+	+	+	+
L. rhamnosus 39V	-	+	-	+	-	-	+	+	+	+
L. rhamnosus 40V	-	+	-	-	-	-	+	+	-	+
L. paracasei 41V	-	+	-	+	-	-	+	+	+	+
L. bulgarcus 42V	-	+	-	+	-	-	+	+	-	+
L. plantarum 43V	-	+	-	+	-	-	+	+	-	+
L. plantarum 44V	-	+	-	-	-	-	+	+	-	+
L. rhamnosus 45V	-	+	-	-	-	-	+	+	-	+
L. plantarum 46V	-	+	-	-	-	-	+	+	-	+
L. rhamnosus 47V	-	+	-	-	-	-	+	+	-	+
L. plantarum 48V	-	+	-	+	-	-	+	+	+	+
L. rhamnosus 49V	-	+	+	-	+	-	+	+	-	+
L. rhamnosus 50V	-	+	+	-	+	-	+	+	-	+

Table 3. Number of viable lactic acid bacteria adhered to mucin.

Strain	Number of bacteria adhered to mucin, CFU mL^{-1}
L. rhamnosus 37V	$5.5 \times 10^4 \pm 0.32$
L. rhamnosus 39V	$8.5 \times 10^5 \pm 0.22$
L. delbrueckii subsp. *bulgaricus* 41V	$1.9 \times 10^3 \pm 0.38$
L. paracasei 43V	$5.4 \times 10^3 \pm 0.48$
L. rhamnosus 46V	$1.2 \times 10^5 \pm 0.80$
L. plantarum 47V	$5.5 \times 10^4 \pm 0.43$
L. koreensis 35V	$1.9 \times 10^6 \pm 0.23$
L. plantarum 48V	$8.1 \times 10^1 \pm 0.36$

**Results are mean ± SD of three different experiments.*

explaining its absence from our strains. The *gtf* was detected in only two *L. fermentum* strains (12V and 23V). *Mub*1, *msa* and *mapA*, were found in 16 %, 16 % and 26 % of LAB strains, respectively, while *mub*2 was found in 72 %. *Cnb*, which encodes a collagen-binding protein and *fpbA,* which encodes a fibronectin binding protein, also shown 100 % presence and were detected in all our strains. The latter gene, which was considered non-essential, has been reported in several pathogenic species and in many LAB species [56, 57]. LAB and pathogens may have comparable binding processes that involve proteins with similar activities. This supports the discovery that some LAB can reduce pathogen adherence to intestinal cells through competition [58]. The *apf*, encoding the aggregation-promoting protein LBA0493, was detected in all strains in our collection. Although there were no significant conserved domains found in this protein, the *apf* gene was widely distributed throughout *Lactobacillus* species [59]. Thus, our results are in accordance with the reported results.

Mucin adhesion assay

Adherence of beneficial bacteria to the intestinal mucosa is considered important for exerting their function and is a claimed key characteristic of probiotics [60]. Probiotic bacteria's adherence to mucin immobilized onto abiotic surfaces (like polystyrene), intestinal cell layer cultures (HT-29, HT-29-MTX, and Caco-2), or extracellular matrix elements (collagen and fibronectin) are frequently examined in in vitro studies [61 - 63].

In this study, we conducted an experiment to test the ability of eight strains of LAB to adhere to mucin, selected according to our previous investigations. The obtained results showed different adhesiveness of the selected strains of LAB on the mucin immobilized in the polystyrene plaques. The number of adherent cells was on the order of 10^1 to 10^6 CFU mL^{-1} (Table 3). Bacterial viability was unaffected by Triton X-100 treatment, even after 30 min of incubation at 37^{o}C. The greatest adhesive ability was observed for *L. koreensis* 35V (1. 9 × 10^6 CFU mL^{-1}). Generally, the degree of adhesion varies both between individual species and between strains belonging to the same species (Table. 3). An example of this is the three strains of *L. rhamnosus* (37V, 39V and 46V) and the two strains of *L. plantarum* (47V and 48V). Strains 46V and 39V (*L. rhamnosus*) showed higher adhesive properties to mucin than strain *L. rhamnosus* 37V. Strain *L. plantarum* 48V showed weaker adhesive properties than strain *L. plantarum* 47V. In a similar study conducted by Dhanani and Bagchi, different degrees of adhesion to mucin were observed for the investigated lactobacilli strains. Among the species studied, the adhesion ability of the *L. delbrueckii* strain and the *L. plantarum* strain was statistically comparable to their reference strain *L. rhamnosus* GG, which proved to be the probiotic strain with the best adhesion properties. In their study, the adhesion ability of *L. fermentum* and *L. casei* was low compared to all other tested strains [64].

These results are like ours, confirming that the adhesion abilities of lactic acid bacteria vary even between strains of the same species.

CONCLUSIONS

In this study fifty strains, representatives of seven LAB species, isolated from different biotopes, were tested for a set of abilities regarding their adhesive and aggregation properties as part of their probiotic potential. The ability of auto-aggregation, co-aggregation, adhesion to mucin and biofilm production were differently represented among the strains. Generally, it has been observed that a great percentage of our LAB strains are strong BFB and can successfully inhibit the biofilm formation of pathogenic bacteria and fungi. Based on the obtained results, it can be assumed that the aggregation and co-aggregation abilities of the lactic acid bacteria isolated by us are strain-specific. Moreover, the longer the contact between LAB and pathogenic microorganisms is, the higher the aggregation capacity of the LAB strains tends to be, thereby potentially reducing the harmful effects exerted by the pathogens.

The results obtained in this study contribute to the enrichment of the knowledge about the LAB biodiversity of our geographical region. Our main observation is that none of the tested strains meets all probiotic criteria simultaneously, but when applied in a complex they can exhibit a significant probiotic effect. The application of complex methods to determine the specific properties of isolated strains will enable a comprehensive in vitro assessment of their biological potential.

Therefore, it is important to study bacterial adhesion in different in vivo models to gain knowledge about the general adhesion ability of probiotic strains. This will be the focus of our future investigations for biotechnological application of potentially probiotic strains.

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