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ORIGINAL RESEARCH PAPER

SELECTION OF NEW LACTOBACILLI STRAINS WITH POTENTIALLY PROBIOTIC PROPERTIES

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Abstract

In recent years, there has been an increasing interest among food industry producers for functional foods. In this context, the study aims to isolate new lactic acid bacteria strains with probiotic potential that could further be used to obtain valuable metabiotics with good technological potential and health benefits. In the current study, twenty-nine newly LAB strains were isolated from various natural sources, including artisanal cultures (like, kefir grains and kombucha membrane), and selected through fermentation potential on unconventional plant-base substrates, consisting red lentils flour, red potato peel powder, and okara. The strains identified using 16S rDNA sequencing, as *Lacticaseibacillus paracasei* (encoded BL13, BL80, BL87) and *Lactiplantibacillus plantarum* (encoded BL21) were further screened considering their *in vitro* probiotic properties. The safety assessment evaluation highlighted that *Lacticaseibacillus paracasei* (BL13) and *Lactiplantibacillus plantarum* (BL21) selected as performant strains have antimicrobial potential against *Escherichia coli* and *Staphylococcus aureus*, being also susceptible to tetracycline, chloramphenicol and ampicillin. Furthermore, *Lactiplantibacillus plantarum* (encoded BL21) proved good adhesion properties on the HT-29 cell line. These strains are new promising probiotic starter cultures candidates with approachable potential to be used for obtaining functional food and ingredients through fermentation processes, that could offer health benefits.

Keywords: wild lactic acid bacteria, artisanal cultures, rDNA 16S sequencing, probiotic potential, safety assessment, health benefits

Introduction

The gut microbiota is critical for the development and maintenance of host physiology and immunity. Low-dose antibiotics are now the most successful technique for modulating this natural microbiome. However, due to the growth of

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multi-drug-resistant bacteria number and their potential to cause human infections, overuse of antibiotics has become a major global issue. As a result, screening of microorganisms with favorable characteristics as antibiotic alternatives has gain a lot of interest on the way to solve this problem (Zhang *et al.*, 2022).

Alternately, lactic acid bacteria (LAB) have received much attention, primarily because of their GRAS status and secondly for the various beneficial effects derived from their bioactive properties (Chen *et al.*, 2020) that allow them to be exploited as metabiotic promoters (prebiotics, probiotics, postbiotics, and paraprobiotics) (Azizian *et al.*, 2021; Divyashree *et al*., 2021)

The probiotics are microorganisms that can improve the human healthiness by modulating the gut barrier function (Inayah *et al.*, 2022). Potential probiotic bacteria can be employed as a biotherapeutic agents and a long-term replacement for antibiotics, as well as an antioxidant, anti-inflammatory, and antidiabetic agents, all without generating major adverse effects (Khan *et al*., 2021). To be employed as probiotics, LAB must possess certain features, such as resistance to gastrointestinal tract environment (Margalho *et al*., 2021), antibiotics susceptibility, and inhibitory action against pathogenic microorganisms (Azizian *et al.*, 2021). According to scientific reports, consumption of probiotics has been found to provide a variety of health benefits (Chen *et al.*, 2020), including antiallergic, anticancer and, cholesterol-lowering effects, to improve the host immune response, prevent and remediate irritable bowel syndrome, the enteritis, and numerous other gastrointestinal disorders (Khan *et al*., 2021; Zhang *et al*., 2022).

Over the years, the number of lactobacilli species increased, more than 260 species being described in 2020 (Zheng *et al*., 2020). Based on the lactobacilli diversity from metabolic, ecological and genetic perspectives, their taxonomy was subjected to a major reevaluation, integrating new genus names and being also grouped into new clades or clusters (Oberg *et al*., 2022). Thus, besides the starter cultures from *Lactobacillus* genus, there were also described nonstarter lactic acid bacteria (NSLAB) that were included into other two new genera designated as *Lacticaseibacillus* (*Lcb*.) and *Lactiplantibacillus* (*Lpb*.). The NSLAB can further be isolated, assessed and used in the fermentation processes for enhancing the properties of the final products (Margalho *et al*., 2021).

Artisanal cultures, such as, kefir grains (water/milk) or kombucha contain multiple wild microorganisms (LAB, acetic acid bacteria and yeasts) associated in natural consortia, which work in a perfect symbiosis, being considered valuable promoters for bioactives production and a great isolation source of probiotics with important characteristics (Cotârleț *et al.*, 2020). LAB isolated from different raw cereals or flours, richness in fiber, proteins and micronutrients, were used in the fermentation processes aiming to obtain fermented food with value-added compounds. Recent trends targeted the valorization of agri-food by-products through fermentation in order to obtain functional fermented products with technological and health benefits, promoting also circular economy principles. So, there is a huge economic impact, if it is considered the conversion of low-cost substrates into high value-added molecules through an elementary fermentation process (Ilango *et al*., 2021).

The raw materials used in this study as unconventional fermentation substrates are innovative for medium formulation. The okara is a by-product derived from of soymilk or soybean curd (tofu) processing, rich in proteins, carbohydrates, fibers, unsaturated lipids, isoflavones and minerals that can be used as substrate for many microorganisms in growth and fermentation process (Cotârleț *et al.*, 2020). On the other hand, red potato peel accumulated in large quantities after processing represents valuable raw material for media formulation with applicability in biotechnology (Javed *et al.*, 2019). Moreover, red lentils are known for their high content in proteins, vitamins, minerals, and carbohydrates, as well as for valuable nutrients for microorganisms` metabolism (Chen *et al.*, 2020).

In the present study, twenty-nine newly LAB strains isolated from several natural environments including kefir grains and kombucha membrane microbiome were tested for their abilities to ferment unconventional substrates so to bring out their biotic properties, and to also emphasize their future applications as valuable probiotics based on the *in vitro* research` results.

Materials and methods

Reagents and cultures

Fetal bovine serum (FBS) and Dulbecco's Modified Eagles Medium (DMEM) are from Merck KGaA, Darmstadt, Germany. L-Glutamine 200 mM (100X), non-essential amino acids solution, trypsin/EDTA and penicillin-streptomycin-neomycin (PSN) antibiotic mixture solutions were purchase from Gibco (ThermoFisher Scientific, USA). The sheep blood used for the haemolytic test was from Oxoid (Deutschland GmbH, Germany). Man, Rogosa and Sharpe agar (MRS) (Sigma-Aldrich, Sweden) medium was used for LAB cultivation, Muller-Hinton (Scharlau, Barcelona, Spain) for *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 25923 growth and Brain Heart Infusion (Oxoid, Hampshire, England) broth or with 1.5% agar (Biolab, Hungary) for *Listeria monocytogenes* ScottA, respectively. The cell line HT-29 (ECACC 91072201) was provided by European Collection of Authenticated Cell Cultures (ECACC) and was used for the LAB adhesion.

The water and milk kefir grains were provided from a Romanian private household collection. Kombucha culture originated from an artisanal manufacturer from Republic of Moldova. Red lentil flour was obtained by grinding red lentils (Lider Company, Chișinău, Republic of Moldova). Sweet potatoes were purchased from a supermarket (Galati, Romania) and were processed according to Marconato *et al.* (2020).

Kefir grains and kombucha propagation

For artisanal cultures propagation, the milk kefir grains were transferred into pasteurized whole milk (3.5% fat), incubated at 25°C for 48 h (Binder BF 400, Tuttlingen, Germany), separated from the milk, and washed with distilled water (Cotârleț *et al.*, 2019). The water kefir grains were propagated by incubation in sugar water (1.0%, w/v) supplemented with raisins 3.0% (w/v), at 25°C for 48 h. The grains

were filtered using a plastic kitchen sieve and rinsed with distilled water (Talib *et al*., 2019). The propagation medium was obtained by boiling the water with sugar 5.0% (w/v). Then, the 0.5% (w/v) black tea was added for infusion approx. 5 minutes. After cooling, the sweet tea was filtered, inoculated with the 3.0% (w/v) kombucha membrane and incubated for 7 days at 30°C (Ayed *et al.*, 2017).

Isolation of lactic acid bacteria

For the new LAB strains isolation, 10g from each natural source samples (Table 1) were suspended in 90 mL of sterile saline solution (0.9% w/v) and homogenized with a pulsifier (PUL 100E, Microgen Bioproducts Ltd., UK) in a sterile bag for 2 min. Subsequently, serial 10-fold dilutions were made, and 0.1 mL was inoculated, in triplicates, by surface spreading on MRS plates containing 10 g/L $CaCO_3$. The pure cultures of isolated LAB strains, were preliminary coded with indicative BL and preserved in 40% (v/v) glycerol at - 80°C.

Medium based unconventional substrates formulation and fermentation

The okara obtainment was carried out by using the method described by (Vong *et al.*, 2019) with modifications proposed by Cotârleț *et al.* (2019). The sweet potatoes peels were lyophilized using Alpha 1–4 freeze-dryer (Martin Christ, Osterode am Harz, Germany), followed by grinding. The fermentation medium based on unconventional substrates has the following composition, % (w/v): red lentil flour - 5, sweet potato peel flour - 4, okara - 3 and distilled water up to 100 mL (pH 6.2). After the medium sterilization at 121°C for 30 minutes, it was cooled and then inoculated with 2% (v/v) of LAB inoculum (OD600=2.0 of an overnight culture in MRS broth) and incubated for 72 h, at 37°C, in a stationary system and aerobiosis conditions.

pH and total titratable acidity evaluation

Total titratable acidity (TTA) of fermented products was assessed using an automatic titrator (TitroLine Easy, Schott Instruments, Mainz, Germany). Thus, 10 g of the fermented sample was homogenized with 90 mL of distilled water. The mixture was titrated with 0.1 N NaOH solution to a final pH of 8.5, and the results were expressed as milliliters of 0.1 N NaOH (Pacularu-Burada *et al.*, 2020).

Lactic acid bacteria identification

For phylogenetic characterization of selected LAB strains, the 16S rRNA gene sequencing (CD Genomics Shirley, NY, USA) using Sanger method and the dendrogram were assayed. The 16S rRNA gene (from genomic DNA) was amplified according to Abushelaibi *et al.* (2020) by PCR (Polymerase Chain Reaction) using 27F (5`-AGAGTTTGATCCTGGCTCAG-3`) and 1492R (5`-GGTTACCTTGTTACGACTT-3`).

The sequencing results were compared with 16S rDNA of other bacterial sequences using BLASTn (blast nucleotide) from NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic analysis was designed using the Neighbor-Joining method in MEGA X software (version 11.0; Biodesign Institute, Tempe, USA) (Kumar *et al*., 2016). The GenBank accession numbers of

the type strains used in the alignments were as follows: NR_113823.1, NR_025880.1, NR_113332.1, NR_025447.1, NR_029133.1 and NR_115605.1.

Probiotic properties evaluation

Tolerance to low pH

The LAB tolerance to low pH was determined according to Adesulu-Dahunsi *et al.* (2018). The biomasses obtained after centrifugation (2879xg, 4°C, for 10 min) (Hettich Universal 320R, Tuttlingen, Germany) of the overnight LAB cultures were washed twice with PBS and resuspended in 50 mL MRS broth having different pH values (pH 2.0 and 3.0). The samples were incubated at 37°C for 4 h. At each hour, samples were taken and plated on MRS agar medium supplemented with 1% (w/v) $CaCO_3$. The plates were incubated at 37°C for 72 h and then, the total viable counts were assayed. The survival rate of the bacteria (%) was calculated using equation 1 (Talib *et al.*, 2019).

$$\text{Survival rate, \%} = (\log \text{CFU/mL } T_X \div \log \text{CFU/mL } T_0) \times 100 \quad (1)$$

where, log CFU/mL T_X - total viable cells concentration survived at pH 2.0, taken at different periods of time; log CFU/mL T_0 - initial inoculated cells` concentration of ~9.0 log CFU/mL.

Bile salts resistance

The ability of the LAB strains to grow in bile salts was performed according to Adesulu-Dahunsi *et al.* (2018). Briefly, the overnight LAB cultures were centrifuged at 2879xg, 4°C, for 10 min and washed twice with PBS (pH 7.4). Further, 50 mL of MRS broth supplemented with 0.3% bovine bile salt (w/v) was inoculated with the obtained bacterial biomass and then incubated at 37°C for 4 h. The samples were taken every hour, plated on MRS agar medium supplemented with 1% (w/v) $CaCO_3$ and incubated at 37°C for 72 h. The survival rate of the LAB strains (%) was calculated using equation 1.

Resistance of isolated LAB strains to simulated gastrointestinal tract conditions (GIT)

The resistance of LAB strains to gastrointestinal simulated conditions was performed according to (Margalho *et al.*, 2021). The gastrointestinal juices were prepared as follows: (i) the simulated gastric juice contains 7 mM KCl, 125 mM NaCl, 3 g/L pepsin and 45 mM $NaHCO_3$ (pH 2.0 adjusted with 0.1 N HCl); (ii) the simulated duodenal juice comprising 1% (w/v) bovine bile, (pH 8.0, adjusted with 10N NaOH) and, (iii) the simulated ileum juice comprising 0.1 % (w/v) pancreatin and 0.3 % (w/v) of bovine bile (pH 8.0). The biomass of LAB was resuspended in 10 mL of sterile gastric juice supplemented with 10 mL of pepsin. This mixture was incubated at 37°C with 200 rpm for 90 min. The samples were taken at the initial time (t_0) but also at every 30 min (T30; T60; T90). Further, the bacterial biomass was centrifuged (2879×g, 10 min, 4°C) and resuspended in 10 mL of simulated duodenal juice. After 10 minutes, the samples were collected for analysis. For the *in vitro* ileum digestion, the bacterial biomass was added in a mixture of 10 mL ileum juice and 10 mL

pancreatic solution, and maintained for 90 min at 37°C, with 200 rpm. The samples collected after 30, 60 and 90 min were plated on MRS agar and incubated at 37°C for 72 h. The survival rate (%) of the bacteria was calculated using equation 1. *Lactobacillus paracasei* ssp. *casei*, a commercial lactic acid bacterium with probiotic properties was used as control strain.

Coaggregation activity

The pathogenic strains, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were grown in Muller-Hinton broth medium at 37°C, for 18 hours (Guan *et al*., 2017). The cells were resuspended in PBS (pH 7.4) after two steps of washing and the optical density of suspension was adjusted to 1.0 (OD600, A0). An equal concentration of LAB cell suspension with either *E. coli* or *S. aureus* cells were mixed and incubated at 37°C for 4 h. The final OD600 values (Af) of the aqueous phase was measured and the percentage of co-aggregation (Aco) was expressed based on equation 2 (Sui *et al*., 2021).

$$\text{Aco, \%} = \frac{\text{A0} - \text{Af}}{\text{A0}} \times 100 \qquad (2)$$

Antimicrobial activity

The antimicrobial activity of LAB cell-free supernatants (CFS) was assessed against *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Listeria monocytogenes* Scott A, in accordance with Daoud *et al*. (2019) with a few modifications. Briefly, 7 mL of MH (Mueller-Hinton) semisolid medium (containing 0.75% (w/v) agar) inoculated with 10 µL from each pathogenic overnight culture was poured on the first layer of agar media, to achieve a concentration of ~ 10^7 CFU/plate. Wells with a diameter of 0.8 cm were made and 100 µL of CFS were added to each well. The CFS was obtained by centrifugation of the fermented medium for 15 min at 5643×g, and 4°C. The plates were incubated at 37°C for 24 hours and the results were expressed in millimeters (mm) after diameters of the growth inhibition zones were measured.

Safety assessment

Blood hemolytic test

Blood hemolytic test was performed according to the protocol of (Sui *et al*., 2021). The LAB strains were streaked on the Columbia agar plates medium containing 5% sheep blood, incubated at 37°C for 3 days, and then examined for the hemolytic reaction. The presence of α- or β-hemolysis is indicated by the formation of greenish or clear zones.

Resistance to antibiotics

Antibiotic resistance was measured using the Kirby-Bauer disk diffusion susceptibility test (Christenson *et al*., 2018). The MRS agar plates were inoculated with 1mL of overnight LAB culture (OD600 of 0.5) and then, different antibiotic discs (6 mm diameter) were aseptically placed on the agar medium surface. The

antibiotics tested were: cotrimoxazole 25 (SXT25), chloramphenicol 30 (C30), tetracycline 30 (TE30), gentamicin 10 (CN10), ofloxacin 5 (OFX5), ampicillin 10 (AM10), amikacin 30 (AK30), levofloxacin 5 (LEV5), ciprofloxacin 5 (CIP5), penicillin G10 (P10) (Biomaxima, Poland), fosfomycin 200 µg (FF200), cofoxitin 30 µg (Fox30) (Bioanalyse, Turkey), vancomycin 5 µg (VA5) and ceftazidime 30 µg (CAZ30) (Oxoid, England). The plates were incubated at 37°C for 48 hours and the inhibition zone diameters (IZD) were measured. The correlation between IZD and the susceptibility of the strains was established by consulting the corresponding tables from the CLSI standard (Sharma *et al.*, 2016). The results were expressed in susceptible, moderately susceptible and resistant.

The epithelial cell line culture and the adhesion assay

The HT-29 cell line was used in order to test the adhesion capacity of the identified LAB strains. Briefly, approx. 10^5 cells of HT-29/mL were grown into 24-well plates (with coverslips) in DMEM medium supplemented with 10% fetal bovine serum (FBS), 1% ultraglutamin, 1% nonessential amino acids and 1% a mixture of antibiotics (50 U/mL of penicillin, streptomycin and neomycin) at 37°C, in an atmosphere with 5% CO_2, until the monolayers are formed. With 24 hours before adding the lactic acid bacteria, the cell monolayers were washed with PBS (pH 7.4) and the medium was replaced with fresh DMEM without antibiotics and FBS. In parallel, the bacterial cells were grown in MRS broth for 24 h at 37°C, washed with PBS (pH 7.4), centrifuged at 3.000xg for 10 min and then resuspended in order to have a suspension with the concentration of approx.10^8 CFU/mL. The LAB inoculum (1 mL) was added to each well, over the HT-29 monolayer and incubated for 3 h at 37°C (Barnett *et al.*, 2018). After incubation, the non-adherent cells were washed with PBS and the adhered bacterial cells from the coverslips were fixed with 1 mL of methanol, stained for 30 minutes with Giemsa solution at room temperature and then, washed with distillate water. The adhesion of the LAB was observed with the Zeiss AxioSkop 40 microscope and the images were recorded with AxioCam MRc. In order to count the LAB cells that have adhered to the epithelial cell line, firstly a treatment for 10 min with trypsin/EDTA was made. Secondly, 1 mL from the bacterial suspension ($1x10^8$ CFU/mL) was resuspended in 9 mL of saline sterile solution and plated on MRS agar after decimal serial dilutions. The plates were incubated for 48 h at 37°C and the results were expressed as CFU/mL.

Production of Nitric Oxide (NO) assay

The HT-29 cells were seeded in standard conditions, for 24 h in order to reach $1x10^5$ cells/mL. The NO synthesis by HT-29 cell line was evaluated with Total Nitric Oxide Assay Kit (Invitrogen, Vienna, Austria) from cell line supernatant, after 4 h of co-cultivation with LAB strains encoded BL13 and BL21, in concentrations of 7 log CFU/mL, 8 log CFU/mL and 9 log CFU/mL, respectively.

Statistical analysis

The experiments were made in triplicate. The data are presented as mean ± standard deviation. Minitab 19 software (Minitab Inc., Pennsylvania, USA) was used to

determine the statistical differences among samples by ANOVA and Tukey test. For p-values < 0.05 the results were considered statistically significant.

Results and discussion

The fermented product attributes

Nowadays, the fermented food and ingredients are produced through fermentation of a large type of substrates and cultures offering technological impact and a number of beneficial health effects. In this work, different natural microbiota from borsh, corn and barley flour, whey, and sesame seeds as well, artisanal cultures (milk kefir grains, water kefir grains and kombucha) were used to isolate twenty-nine wild LAB strains that were screened, firstly considering their specific morphology, and different phenotypical characteristics (data not shown) (Table 1).

Table 1. Acidification potential (pH and TTA) of newly isolated LAB strains after 72 h of fermentation at 37°C, inoculated into medium with red lentil flour, sweet potato peel flour and okara.

LAB strain code	pH	TTA, mL	Natural microbiota used for wild
BL2	3.98±0.02	11.05±0.12	sourdough
BL3	3.93±0.11	11.87±0.15	Barley flour
BL4	3.56±0.01	15.47±0.02	Borsch
BL13	3.57±0.12	16.01±0.03	Corn flour
BL21	3.57±0.08	14.61±0.11	Corn flour
BL24	3.55±0.03	16.10±0.02	Whey
BL35	3.99±0.01	9.11±0.10	Milk kefir grains
BL37	5.57±0.01	16.02±0.01	Water kefir grain
BL38	4.58±0.11	4.83±0.17	Chickpea
BL40	4.08±0.03	7.45±0.08	Sesame seeds
BL72	4.72±0.01	4.27±0.05	Durum wheat flour
BL73	3.58±0.02	11.25±0.03	Sour pickles
BL74	3.54±0.01	16.82±0.14	Sour pickles
BL77	3.69±0.01	12.57±0.28	Water kefir grains
BL78	3.61±0.04	14.71±0.15	Water kefir grains
BL79	3.71±0.01	15.72±0.11	Water kefir grains
BL80	3.59±0.02	16.01±0.23	Water kefir grains
BL81	3.60±0.11	14.71±0.13	Water kefir grains
BL82	3.73±0.15	14.88±0.01	Milk kefir grains
BL83	4.10±0.02	8.43±0.03	Milk kefir grains
BL84	4.60±0.13	4.87±0.17	Kombucha membrane
BL85	4.29±0.11	6.39±0.26	Milk kefir grains
BL86	3.66±0.06	14.80±0.21	Milk kefir grains
BL87	3.63±0.02	15.56±0.17	Milk kefir grains
BL88	3.66±0.01	14.43±0.09	Milk kefir grains
BL89	3.66±0.11	15.56±0.01	Milk kefir grains
BL90	4.13±0.05	8.56±0.25	Milk kefir grains
BL91	4.24±0.07	6.63±0.05	Milk kefir grains
BL92	4.25±0.03	6.43±0.20	Milk kefir grains

The experiments were designed to have an accurate selection of the LAB strains that could exhibit probiotic properties. A good fermentation potential was assigned to a few strains isolated from water kefir grains (coded as, BL37, BL77, BL78, BL79, BL80, BL81), milk kefir grains (BL82, BL86, BL87, BL88, BL89), sour pickles (BL74, BL73), whey (BL24), corn flour (BL21, BL13), borsch (BL4), barley flour (BL3) and sourdough (BL2), strains that highlighted a broad spectrum of high total titratable acidity (TTA) values ranging from 11.05±0.12 mL of 0.1 N NaOH to 16.82±0.14 mL of 0.1 N NaOH, with pH values below 3.98±0.02 (Table 1). For the rest of the isolated strains (BL35, BL38, BL40, BL72, BL84, BL85, BL90, BL91 and, BL92) the TTA values were significant lower, ranging from 4.27±0.05 mL of 0.1N NaOH to 9.11±0.10 mL of 0.1 N NaOH and with a pH higher than 3.99±0.01. Considerable TTA variations were detected among the LAB species, aspects that could be associated with the strains` isolation environments (Table 1). Different TTA values were reported in the literature being associated with the experimental conditions, especially with the media composition and the nutrients availability (Omedi *et al*., 2019; Păcularu-Burata *et al*., 2020; Păcularu-Burata *et al*., 2021).

In the fermentation process, organic acids such as, lactic, acetic, butyric, or propionic acids, etc. are synthesized in correlation with a decreased pH and an increased TTA , aspects that depend on the intrinsic and extrinsic factors of the biological bioprocesses. Therefore, the TTA evaluation of the LAB-fermented products was treated as an important screening criterion considering that a higher TTA value indicated strains with good fermentative properties, including the capacity to synthesize valuable short-chain organic acids, or different metabolites as post-biotics, tolerance to acidic environments and, anti-pathogenic activities (Wegh *et al.*, 2019; Sun *et al*., 2020).

Addressing the fermentation properties, nine of the strains encoded as, BL1, BL4, BL13, BL21, BL24, BL38, BL74, BL80, BL87, were preliminary selected for genetic identification.

Molecular identification of selected LAB strains

The BLAST function on NCBI database showed that all the analyzed strains were part of *Lactobacillaceae* family having a high degree of similarity (>99%) with the following strains: *Lcb. paracasei*, for the strains encoded as BL1, BL4, BL13, BL80, BL87, *Lpb. paraplantarum* for the strain encoded as BL74, *Lpb. plantarum* for the strain encoded as BL21, *Lpb. pentosus* for the strain encoded as BL24, and *Lcb. rhamnosus* for the strain encoded as BL38 (Table 2).

Figure 1 shows the Neighbor-Joining tree of the nine LAB strains' 16S rDNA sequences. In the phylogenetic analysis, the 16S rDNA sequences of the following type strains were also included: *Lactobacillus paracasei* subsp. *tolerans* strain NBRC 15906, *Lactobacillus paracasei* strain R094, *Lacticaseibacillus rhamnosus* strain NBRC 3425, *Lactiplantibacillus paraplantarum* strain DSM 10667, *Lactobacillus pentosus* strain 1242 and *Lactiplantibacillus plantarum* strain JCM 1149.

Table 2. The identity percent of studied LAB 16S rDNA sequences with correspondent sequences from NCBI.

Strains	Identity percent	Identified strains as the BLAST* result	Accession number
BL1	99.91%	*Lacticaseibacillus paracasei*	MT545033.1
BL4	99.79%	*Lacticaseibacillus paracasei*	MT626061.1
BL13	99.86%	*Lacticaseibacillus paracasei*	MT611827.1
BL21	99.33%	*Lactiplantibacillus plantarum*	MW617265.1
BL24	99.65%	*Lactiplantibacillus pentosus*	OK325938.1
BL38	99.58%	*Lacticaseibacillus rhamnosus*	MT463821.1
BL74	99.92%	*Lactiplantibacillus paraplantarum*	MT604712.1
BL80	99.62%	*Lacticaseibacillus paracasei*	CP098411.1
BL87	99.92%	*Lacticaseibacillus paracasei*	ON631824.1

*BLAST – Basic Local Alignment Search Tool

Even though the substitution patterns were homogeneous among species, the compositional distance was correlated with the number of differences between sequences. The tree shows two clades having on one side clearly separated branches with *Lcb. paracasei* species and *Lcb. rhamnosus* and on the other side, the branches with *Lpb. paraplantarum*, that is attached to the cluster formed by *Lpb. plantarum* and *Lpb. pentosus*. The GenBank accession numbers of the LAB 16S rDNA sequences that matched with the queries are mentioned in Table 2.

Moreover, the alignments of the 16S rDNA sequences of the LAB isolates with the similar sequences of the type strains included in the phylogenetic analysis, showed that five of the strains (BL1, BL4, BL13, BL80 and BL87) were grouped alongside the *Lcb. rhamnosus* (BL38) into *Lcb. casei* group member. On the other hand, the *Lpb. plantarum* (BL21) strain was clustered with the type strain, *Lpb. plantarum* JCM 1149, alongside the *Lpb. pentosus* (BL24), *Lpb. paraplantarum* (BL74) and their similar type strains into *Lpb. plantarum* group member. Furthermore, the 16S rDNA sequence of the *Lpb. plantarum* (BL21) was similar with that of the type strain, *Lactiplantibacillus plantarum* JCM 1149, having a bootstrap value of 100%. In any case, the difficulty to identify the *Lcb. paracasei* strains at subspecies level is still an important issue that can be solved by different genetic approaches such as Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD) or ribotyping analysis (Hajigholizadeh *et al.* 2020; Sharma *et al.*, 2020).

Corelating the identification` results with the fermentation properties exhibited by the isolated LAB strains, four of them, namely *Lcb. paracasei* (BL13, BL80, BL87) and *Lpb. paraplantarum* (BL21), were chosen for testing further their probiotic characteristics. The biotic properties of both *Lcb. paracasei* strains (BL80 and BL87) isolated from kefir grains were studied compared to *Lcb. paracasei* (BL13) and *Lpb. plantarum* (BL21) strains isolated from a common substrate, like corn flour.

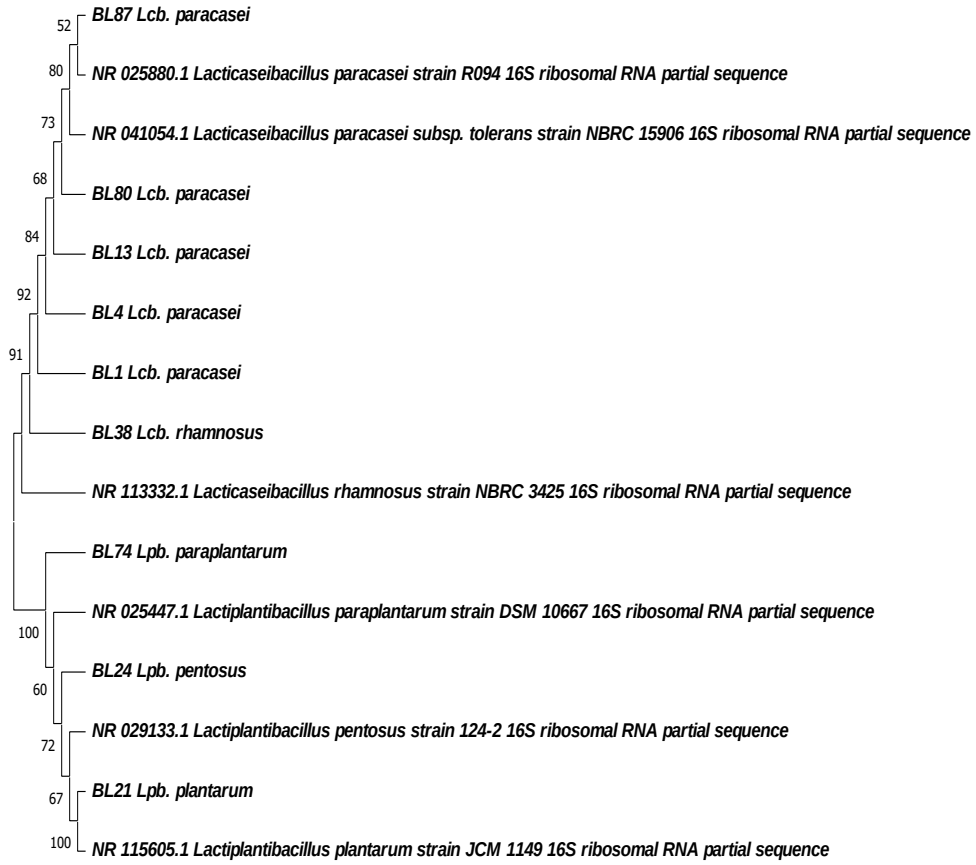

Figure 1. Phylogenetic tree of LAB strains identified based on 16S rDNA sequences. The phylogenetic tree was made using Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA11.

Resistance to low pH and bile salt tolerance

The probiotic potential of a microbial strain depends on its capacity to survive and grow *in vivo* specific environments, such as gastrointestinal tract, where the physicochemical conditions are not optimal for it (Dowarah *et al.*, 2018). In the light of these information, it was tested the tolerance to low pH values, acidic and bile salt environments, as a possible probiotic characteristic of the strains identified as *Lcb. paracasei* (encoded as BL13, BL80 and BL87) and *Lpb. plantarum* (encoded as BL21).

The selected strains ability to survive in media with acidic pH during incubation at 37°C for 4h is depicted in Figure 2A and Figure 2B. At pH 2.0, *Lpb. plantarum* (BL21) strain highlighted a good survival rate compared to the rest of the studied

strains which exhibited a low adaptability, having their viability under 50% at the end of the incubation time. More precisely, *Lpb. plantarum* (BL21) strain after 3h of exposure at pH 2.0 showed high viability rate (84.26%) and also, exhibited a reduction with 0.86%, after 120 minutes of growth in media with pH 3.0. Relatively, Won and co-workers (2021) tested *in vitro*, under similar conditions, the tolerance of *Lpb. plantarum* MG4229, MG4296 and MG5025 strains, indicating a survival rate below 69.5%.

As presented in figure 2B, after 4 hours of growth at pH 3.0, the lowest percent of survivability (95.94%) was for *Lcb. paracasei* (BL80) and the highest (99.87%) for *Lcb. paracasei* (BL87), respectively. In addition to the low pH adaptability, the *Lpb. plantarum* (BL21) strain exhibited a good tolerance to 0.3% bile salt, concentration that had also been shown to be adequate to test the probiotic features (Sharma *et al.*, 2017). If the tolerance to bile salt of *Lpb. plantarum* (BL21) and *Lcb. paracasei* (BL80) strains were similar, having a survival rate of 91.15%, the *Lcb. paracasei* (BL13) and *Lcb. paracasei* (BL87), would display a lower tolerance with 5.28% and 17.10%, respectively (Figure 2C). The distinct behavior of the *Lcb. paracasei* strains observed after their simulated passages from the acidic gastric juice to the basic duodenal environment, highlighted the LAB intra-species heterogeneity and also their osmotolerance variability. Similar observations were mentioned by Reale *et al.* (2015) for a number of strains, such as, *Lb. casei*, *Lb. paracasei* and *Lb. rhamnosus* respectively. The LAB strains tolerance to different bile salt concentrations was explained and proved by applying the CRISPR-Cas9 gene editing, as a new and effective approach to construct different *bsh* (bile salt hydrolase encoding gene) knockout strains starting from *Lactobacillus plantarum* AR113 (Xiong *et al.*, 2017). The viability of *bsh*1 and *bsh*3 genes deletion strains decreased considerable when they were exposed to bile salt contrary to the wild *Lactobacillus* sp. type, thus highlighting the importance of genes regulation process under bile salt stress conditions (Xiong *et al.*, 2017; Wang *et al*., 2021).

LAB behavior in simulated gastrointestinal juices

In vivo, at low pH values of 2.0 or less, attributed to the gastric juice in different digestion stages, the LAB metabolism is significantly affected. First, the resistance of the LAB strains through the harmful influence of chlorohydric acid and pepsin was tested, in simulated conditions (Figure 2D). The collected data proved that the viability of *Lcb. paracasei* strains, encoded as BL13, BL80, and BL87 was not affected by the acid environment, being higher than 95% on the first 90 minutes of the simulated gastric digestion. Instead, the *Lcb. plantarum* (BL21) strain was less resistant, its viability decreased to 54.88 % under the same conditions. When the cells were exposed to simulated duodenal conditions (1% bovine bile action for 10 min), a viability reduction with 4.24%, 6.02% and 9.53% was further observed for *Lpb. plantarum* (BL21), *Lcb. paracasei* (BL80) and *Lcb. paracasei* (BL87) strain, respectively (Figure 2D).

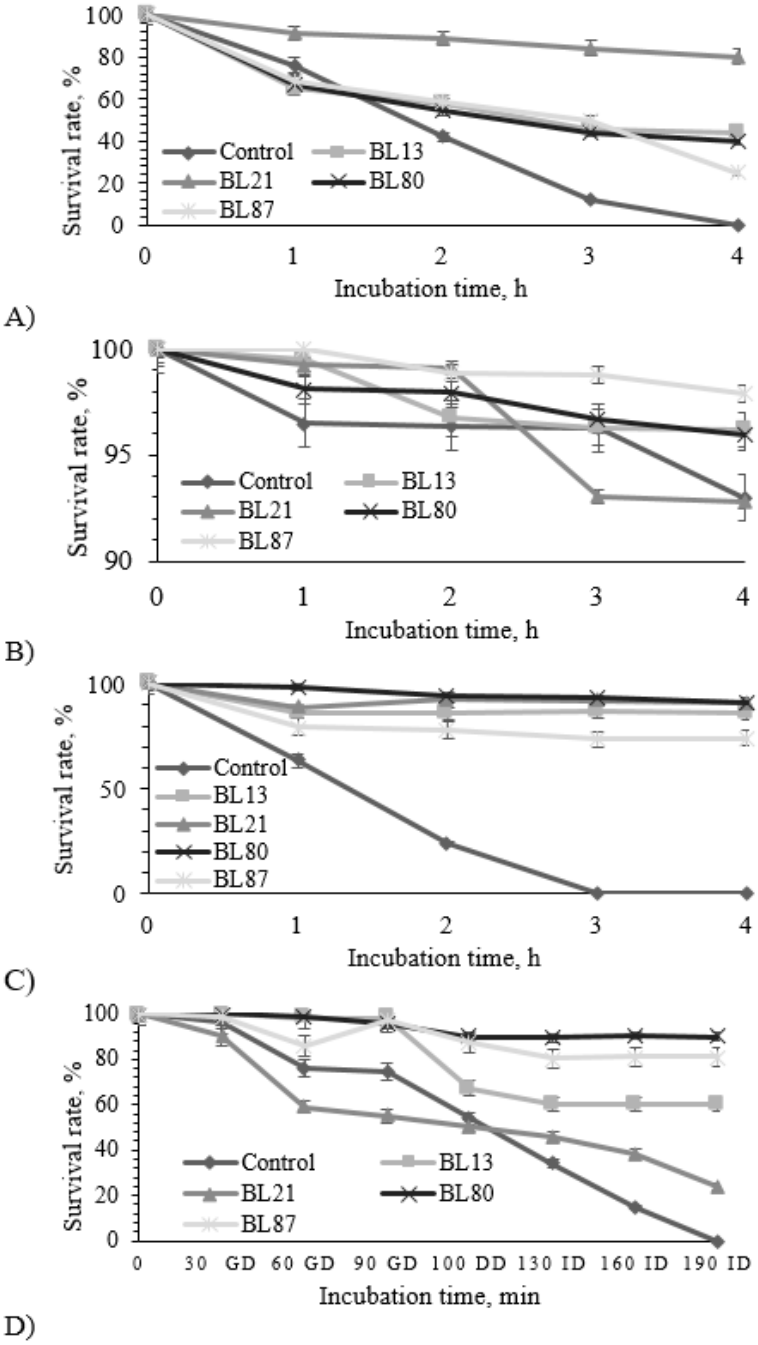

Figure 2. Viability (%) of LAB strains survival at pH 2.0 (A), pH 3.0 (B), 0.3% bovine bile salt during 4 hours (C) or in simulated gastrointestinal juices, during 190 minutes (D). GD-gastric digestion, DD – duodenal digestion, ID – ileum digestion. As the control strain, a commercial *Lactobacillus paracasei* ssp. *casei* culture was used. Data are presented as mean ± SD of three replicates.

Furthermore, the viability of *Lcb. paracasei* (BL13) decreased with 31.2%, compared to the other two *Lcb. paracasei* strains. Moreover, during the first 30 minutes of survival under ileum stress condition (0.3% bile salt and 0.1% pancreatin) only a slightly viability reduction was noticed, that was further maintained, to 89% for *Lcb. paracasei* (BL80) and 81% for *Lcb. paracasei* (BL87) strains respectively. In regard to *Lcb. paracasei* (BL13) and *Lpb. plantarum* (BL21) strains, their survivability decreased more to 60.1% and 23.94%, respectively till the end of the simulated ileum digestion (Figure 2D). da Silva and colleagues (2021) showed that the viability of a few commercial probiotic *Lactobacillus* spp. strains decreased with 1 log CFU/g to 3.6 log CFU/g after gastrointestinal simulation. In our study, a similar viability reduction under *in vitro* gastrointestinal conditions was observed, in case of the *Lcb. paracasei* strains encoded as BL80 (0.77 log CFU/mL), BL87 (1.75 log CFU/mL), and BL13 (3.79 log CFU/mL), respectively. Anyway, the main aspect that is pointed out in the literature is the LAB metabolic ability to survive to a minimum concentration of 6 log CFU/g during its transit through gastrointestinal environment, in order to consider them as health benefits providers (Ranadheera *et al*., 2019; da Silva *et al*., 2021; Prestes *et al*., 2021). The results obtained in the current study highlighted that *Lcb. paracasei* (BL13, BL80, BL87) and *Lpb. plantarum* (BL21) strains were able to survive to a final concentration of 9 log CFU/mL and approx. 7 log CFU/mL during 4 hours in harsh conditions of pH 3.0 and bile salt, respectively.

Coaggregation test

The coaggregation assay was further conducted to evaluate the potential of the four selected strains (encoded as BL80, BL87, BL13 and BL21) to prevent *Staphylococcus aureus* and *Escherichia coli* from surface colonization. Coaggregation is a parameter that highlights the capacity of the LAB strains to adhere to the host tissue, especially to the intestinal epithelium and confers various information about their ability to prevent pathogenic bacteria colonization or to inhibit their growth via antimicrobial compounds. The coaggregation percentages of the tested strains are presented in Table 3. All the LAB strains showed coaggregation ability, but a good percentage was calculated for the strains encoded BL80 (18.76 ±0.07%) and BL13 (19.18 ± 0.20 %) respectively, which exhibited their activity in a mixed suspension with the enteropathogenic *Escherichia coli* strain, after 4 hours of incubation at 37°C. The LAB coaggregation capacity against the same pathogenic bacterium was significantly different ($p<0.05$). Different coaggregation percentages, from 0% to 55.3%, were also reported for a number of strains belonging to *Lb. plantarum* with *Staphylococcus aureus* (Rodríguez-Sanche *et al*., 2021).

Table 3. Coaggregation (%) of LAB cells with Escherichia coli and Staphylococcus aureus during 4h of incubation at 37°C.

Strains	Coaggregation values, %	
	Escherichia coli **ATCC 25922**	***Staphylococcus aureus*** **ATCC 25923**
BL13	19.18 ± 0.20^{aA}	15.19 ± 0.01^{aB}
BL21	11.61 ± 0.06^{dA}	10.06 ± 0.08^{dB}
BL80	18.76 ± 0.07^{bA}	14.00 ± 0.18^{bB}
BL87	12.55 ± 0.05^{cA}	10.44 ± 0.04^{cB}

Data are presented as mean ± SD in triplicate. Different lowercase letters (in a column) denote significant differences for the same pathogen and different LAB strains, whereas different uppercase letters (in a row) denote significance ($p<0.05$) for different pathogens using the same LAB strain.

Antibiotic resistance

Regularly, the antibiotics are co-administrated with probiotics in order to prevent the imbalance of the human intestinal microbiota. On the other hand, a mandatory requirement for the probiotic is the absence of extra-chromosomal genes that can provide and transfer the antibiotic resistance to other bacteria (Binda *et al*., 2020). In general, all tested bacterial strains exhibit susceptibility to tetracycline, ampicillin and chloramphenicol and resistance to a broad spectrum of antibiotics, such as penicillin, gentamicin, amikacin, ciprofloxacin, levofloxacin, ceftazidime (exception being the strain encoded BL13), cefoxitin, fosfomycin, vancomycin, delafloxacin, and cotrimoxazole (except for the strain encoded BL21) (Table 4).

In this regard, the *in vitro* obtained results highlighted the resistance of the selected strains to a wide range of antibiotics, specifically aminoglycosides, fluoroquinolone, and cephalosporins including vancomycin, a matter drawing public attention in the recent years (Montassier *et al*., 2021). The antibiotic resistance to gentamicin, kanamycin, ciprofloxacin, amoxicillin, and vancomycin was also described for different potentially probiotic *Lactobacillus* spp. strains (Won *et al*., 2020; Lin *et al*., 2022). Similar results with those obtained in this study, were debated in the scientific literature suggesting that encoded genes for the antibiotic resistance were found on elements that share extranuclear genetic material (Campedelli *et al*., 2019; Das *et al*., 2019; Fatahi-Bafghi *et al*., 2022). The tetracycline and chloramphenicol genes were reported to be located on the mobile genetic elements or plasmids and, consequently transmissible from these lactobacilli to other strains from the intestinal tract (Fatahi-Bafghi *et al*., 2022). However, this is not the case for neither of the tested strains because all were considered to be susceptible to tetracycline, chloramphenicol and ampicillin (Table 4), according to CLSI standard interpretation.

Linking the in *vitro* survivability of the tested LAB strains in acidic and bile salt conditions with the coaggregation abilities and antibiotic susceptibility, two of them, *Lpb. plantarum* (BL21) and *Lcb. paracasei* (BL13) strains were further screened for more probiotic features. Even though the observed antibiotic susceptibility pattern was similar for all tested LAB strains, nonetheless the *Lcb. paracasei* (BL87) strain resistance to ampicillin and the *Lpb. plantarum* (BL21) sensibility to co-trimoxazole

respectively, contributed to the selection of *Lpb. plantarum* (BL21) and *Lcb. paracasei* (BL13) strains for further analysis.

Table 4. Antibiotic susceptibility of tested LAB strains.

Antibiotics	Diameter of inhibition zone (mm)/ Antibiotic susceptibility* of the LAB strains			
	BL13	**BL21**	**BL80**	**BL87**
Penicillins class				
Penicillin G10 /P	17/R	0/R	15/R	14/R
Aminopenicillins class				
Ampicillin 10 /AM	19/S	22/S	17/S	11/R
Tetracyclines class				
Tetracycline 30 /TE	23/S	15/MS	25/S	22/S
Aminoglycosides class				
Gentamicin 10 /CN	0/R	0/R	0/R	0/R
Amikacin 30/AK	0/R	0/R	0/R	0/R
Chloramphenicol class				
Chloramphenicol 30/C	23/S	23/S	22/S	21/S
Fluoroquinolones class				
2nd generation				
Ciprofloxacin 5/CIP	11/R	0/R	11/R	9/R
Levofloxacin 5/LEV	12/R	0/R	12/R	11/R
Ofloxacin 5/OFX	9/R	0/R	11/R	7/R
3th generation				
Ceftazidime 30 /CAZ	16/MS	0/R	11/R	15/R
Cephalosporins class				
2nd generation				
Cefoxitin 30 /Fox	0/R	14/R	0/R	0/R
Other				
Fosfomycin 200 /FF	0/R	0/R	0/R	0/R
Vancomycin 5 /VA	0/R	0/R	0/R	0/R
Co-trimoxazole 25/ SXT	0/R	21/S	0/R	0/R

*R-resistent, S-Susceptible, MS- moderately susceptible

Antibacterial activity

Table 5 illustrates the antibacterial activity against *E. coli*, *S. aureus* and *L. monocytogenes*, respectively, of the cell-free supernatants obtained by fermentation processes with *Lcb. paracasei* (BL13) and *Lpb. plantarum* (BL21) strains in both media, MRS and media based on unconventional substrates (red lentil flour, sweet potato peel flour, and okara).

Table 5. Antibacterial activity of the cell free supernatants (CFS) of the selected LAB strains.

	Inhibition zone, mm	
LAB stain	BL13	BL21
	CFS from unconventional substrates fermentation	
S. aureus	12.15 ± 0.10^{aA}	10.33 ± 0.05^{bA}
E. coli	15.00 ± 0.10^{bA}	16.03 ± 0.11^{aA}
L. monocytogenes	ND	ND
	CFS derivated from MRS broth fermentation	
S. aureus	10.16 ± 0.05^{aB}	9.53 ± 0.057^{bB}
E. coli	14.33 ± 0.057^{aB}	14.10 ± 0.10^{bB}
L. monocytogenes	ND	ND

Data are presented as mean ± SD of three replicates; ND-not detected; CFS – cell free supernatant. The negative control was represented by the unfermented culture medium, the positive control for *S. aureus* and *L. monocytogenes* was erythromycin (10 mg/mL) and the ampicillin (10 mg/mL) for *E. coli*, respectively. Lowercase letters denote significant differences considering different LAB strains and the same pathogenic bacteria, whereas uppercase letters denote significant differences ($p<0.05$) between the same LAB strains for the same pathogenic bacteria but in different media.

Particularly, when the selected LAB strains were used to ferment the unconventional medium, their supernatants activity against *E. coli* was significantly different ($p<0.05$), causing an inhibition zone diameter of 15.00 ± 0.10mm and 16.03 ± 0.11mm for *Lcb. paracasei* (BL13) and *Lpb. plantarum* (BL21), respectively. Also, the anti-*E. coli* activity of the fermented MRS broth medium compared to the plant-based substrates one, was significantly lower ($p<0.05$). Overall, the antimicrobial activity of the supernatants resulted from fermentation process of the vegetal substrates with both selected strains was higher compared to those obtained after MRS medium fermentation ($p<0.05$). The postbiotics compounds, such as bioactive substances, organic acids, small peptides, that were synthesized during the fermentation processes were the main factors responsible for the growth inhibition of pathogenic bacteria. None of the fermented products obtained proved activity against *L. monocytogenes.*

Hemolytic activity

Another important requirement in the selection of a probiotic strain is the lack of hemolytic activity, as part of the safety evaluation. In this study, none of the selected and tested LAB strains displayed γ-hemolysis, after 72 hours of cultivation on sheep blood agar medium, the results being in agreement to those reported before (Sakoui *et al*., 2022).

LAB strains adhesion and their influence on HT-29 cell line

Besides their antimicrobial activity against pathogenic bacteria, the probiotics should also possess the ability to adhere to the intestinal epithelium and to inhibit or displace the pathogen adhesion by competition (Monteagudo-Mera *et al*., 2019). Microscopic results showed that the *Lpb. plantarum* (BL21) strain exhibited good adhesion

properties being able to adhere to HT-29 cell line (Figure 3A-D) and to maintain a good viability of 6.87 log CFU/mL compared to *Lcb. paracasei* (BL13), whose viability decreased significatively to approx. 4 log CFU/mL ($p<0.05$), after 3h of co-incubation (Figure 3E).

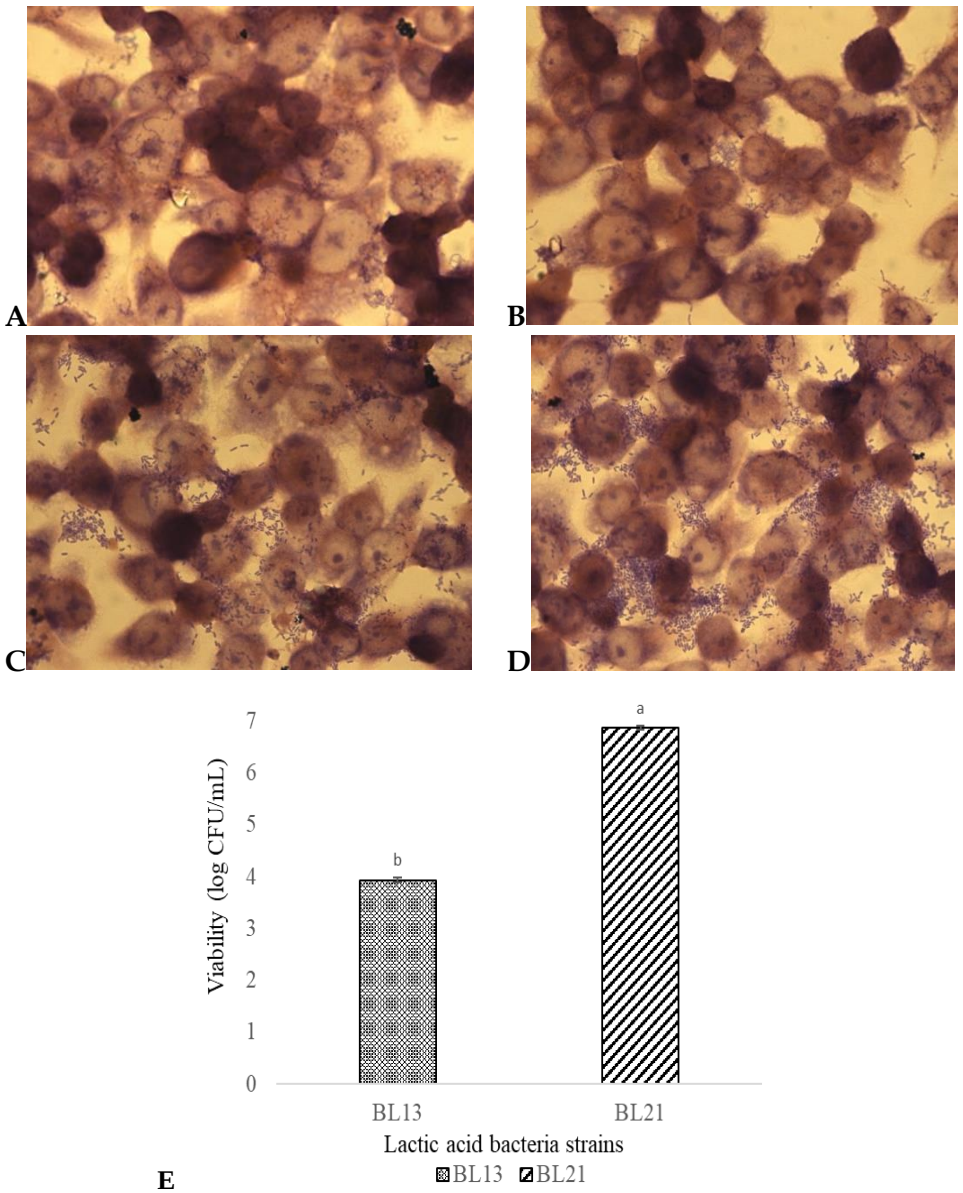

Figure 3. The adhesion of BL13 (A, B) and BL21 (C, D) cells on HT-29 cell line, after 3 hours of incubation. Viability (CFU/mL) of BL13 and BL21 cells adhered to HT-29 epithelial cell line (E). Data are presented as mean ± SD of three replicates. Different letters denote significant differences ($p<0.05$).

Nitric oxide synthesis in HT-29 cell line

Nitric oxide (NO) is a pleiotropic regulator for multiple and essential biological functions. The HT-29 treatment with 9 log CFU/mL of *Lcb. paracasei* (BL13) strain led to a slight but significant ($p<0.05$) NO increment comparing to the cell line treatment with 7 or 8 log CFU/mL, after 4 h of incubation (Figure 4). At 9 log CFU/mL, for both LAB strains studied, the NO synthesis was not significatively different compared to the control. As for the *Lpb. plantarum* (BL21) strain, none of the concentrations used in the experiment affected the NO production in the HT-29 comparing to the untreated cells (Figure 4).

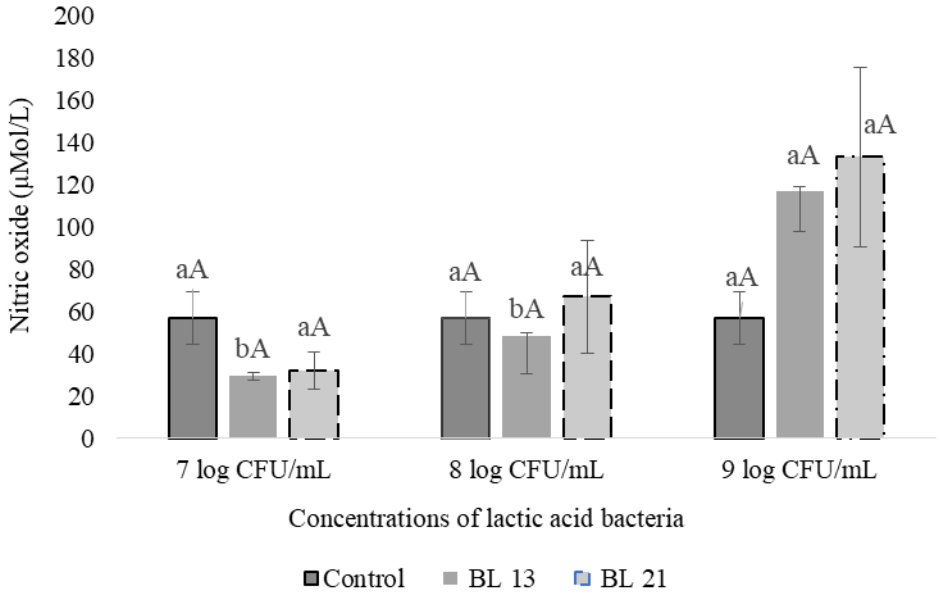

Figure 4. Nitric oxide synthesis that occurs in HT-29 cell line after 4 hours of co-incubation with BL13 and BL21 cultures, in different concentrations. The control was represented by the untreated cells. All data are presented as mean ± SD of two replicates. Different lowercase letters denote significant differences considering the same LAB strain at different concentrations, whereas different uppercase letters denote significant differences ($p<0.05$) between different LAB strains at the same concentration.

On the other hand, the NO production was described before as an endogenous molecule involved in a few essential physiological processes (Vahora *et al*., 2016) and being controversially considered as a pro- or anti-apoptotic inductor, depending on the lactic acid bacteria strains, their concentrations or their types of biological active molecules secreted in the supernatant. Chen and co-workers (2017) proved the pro-apoptotic effect of NO after 4h treating the HT-29 cells with different supernatant concentrations from *Lactobacillus* strains (BCRC 17010, BCRC 14625 PM 153). Further, the authors (Chen *et al*., 2017) emphasized that non-significantly

differences in NO synthesis compared to the control (after 4h) were observed, when the HT-29 treatment was made just with cells of *Lactobacillus* sp. (in concentrations of 10^7CFU/mL, 10^8CFU/mL, and 10^9CFU/mL, respectively). The results obtained in this experiment emphasized that *Lpb. plantarum* (BL21) strain at 10^7CFU/mL and 10^8CFU/mL, respectively and both LAB strains at 10^9CFU/mL did not have any influences on NO synthesis after 4h of co-incubation with HT-29 cells, being in accordance with those reported before.

Conclusions

New LAB strains with potentially probiotic properties were isolated from natural microbiota of different environments and sources, including the artisanal cultures. The selected strains *Lpb. plantarum* (BL21) and *Lcb. paracasei* (BL13) highlighted potentially probiotic characteristics, including their ability to survive under simulated gastrointestinal conditions, good coaggregation capacity with *Staphylococcus aureus* and *Escherichia coli*, in line with their antipathogenic activity, antibiotic susceptibility and *in vitro* adhesion ability on HT-29 cell line. The LAB strains that were isolated from artisanal microbiome of kefir grains and kombucha membrane demonstrated stability of their metabolic activity and single culture viability. Discovering new microorganisms with important probiotic properties offer microbiological and biotechnological heterogeneity as well, based on their secreted bioactive compounds, original fermented products with technological, functional and health impact. So, further *in vivo* tests are necessary in order to fulfill their probiotic potential cumulated with their possibility to be used in food, feed, bioingredients and nutraceuticals formulation that would increase health-related quality of life.

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