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Autochthonous Human Milk Lactobacilli Strains as Potential Probiotic Starter Cultures

Running head: Human Milk Lactobacilli - Potential Probiotic Starter Cultures

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SUMMARY

Research background. Human milk is enriched with bioactive molecules and beneficial bacteria that contribute to shaping the newborn's microbiota. In this study, we aimed to evaluate lactic acid bacteria strains isolated from human milk of healthy Croatian women as potential functional starter cultures.

Experimental approach. In order to define novel potential probiotics for use in dairy products, eight lactobacilli strains were analysed for their proteolytic, antimicrobial and antioxidant activity as well as their survival rate during freeze-drying.

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Results and conclusions. Based on the results obtained, the exopolysaccharide-producing *Limosilactobacillus fermentum* MC1, the surface (S)-layer-producing *Levilactobacillus brevis* MB2 and the plantaricin-producing *Lactiplantibacillus plantarum* MB18 strains are candidates for the production of fermented dairy products with potential functional and nutritional relevance for the host. The selected strains exerted high casein degradation capacity, a broad spectrum of antimicrobial activity and a promising 2,2-diphenyl-1-picrylhydrazyl hydrate radical scavenging activity. They also fulfilled the primary technological criterion by having a high survival rate during freeze-drying.

Novelty and scientific contribution. The data presented emphasise the importance of human milk as a valuable source of lactic acid bacteria with unique technological and functional properties, which are important both as a basis for scientific research and for the development of novel starter cultures for functional products.

Keywords: human milk; microbiota; functional starter cultures; lactic acid bacteria; functionality

INTRODUCTION

Human milk is a source of numerous bioactive molecules that are crucial for the protection and development of the infant. Although it was originally considered sterile, accumulating evidence suggests that it comprises not only biomolecules but also the human milk microbiota, which is of great importance as its disruption, particularly in early childhood, may underlie the development of certain diseases (*1*). Lactic acid bacteria (LAB) with probiotic properties have been isolated from many different sources, but those from human milk are considered valuable because of their human origin and safety for infants (*2*). The most abundant bacterial species in the human milk microbiota belong to the genera *Staphylococcus, Streptococcus, Lactobacillus, Pseudomonas, Corynebacterium, Bifidobacterium, Enterococcus, Rothia, Acinetobacter, Veillonella , Cutibacterium,* and *Bacteroides* (*3,4*). Lactobacilli strains, especially those isolated from fermented milk products, have a highly efficient proteolytic system which ensures self-sufficiency in free amino acids, but is also important for the production of casein-derived bioactive peptides (*5*,*6*). Various LAB strains show antagonistic activity against certain pathogenic microorganisms, which is often attributed to two mechanisms of action (*7*). Firstly, LAB produce organic acid through fermentation, which lowers the pH of the environment and prevents the survival of some pathogens that are intolerable to acidic conditions (*8*). Secondly, LAB can produce bacteriocins, polypeptides that inhibit certain food-borne pathogens and harmful bacteria such as *Escherichia coli*, *Staphylococcus*, *Salmonella* and *Listeria monocytogenes* (*9*,*10*). LAB can also produce surface (S)-layer proteins on their cell surface, which play an important role in the probiotic properties of the producer strain, such as protection against unfavourable

environmental conditions, aggregation capacity and adhesion (*11*). Exopolysaccharides (EPSs), high molecular mass carbohydrate polymers, also contribute to the probiotic properties of LAB by favourably influencing their survival during freeze-drying, adherence to human epithelial cells and competitive exclusion of pathogens (*12*).

Numerous health issues such as diabetes, cancer and cardiovascular, neurological and inflammatory disorders are associated with oxidative stress. Probiotics have garnered growing scientific attention due to the ongoing search for natural antioxidants. Although various LAB strains are currently used in numerous dietary supplements, their antioxidant effects are still relatively poorly understood (*13*). For these reasons, LAB with desirable probiotic properties are being used to develop functional beverages that can improve the health of the gastrointestinal tract of consumers by preventing the proliferation of pathogens and activating the immune system (*14*). Some probiotic strains of lactobacilli and bifidobacteria are frequently used as starter and co-starter cultures for the production of various functional products. They tolerate the low pH of products such as fermented milk during fermentation and cold storage (*15*). Therefore, this work focused on the selection of proteinase-producing, antimicrobial and antioxidant LAB strains previously isolated from the human milk microbiota (*3*) and characterised as potential probiotics (*3*,*12*) to be utilised as functional starter cultures in the production of probiotic products.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions

The bacterial strains analysed in this study are deposited in the Culture Collection of the Laboratory for Antibiotic, Enzyme, Probiotic and Starter Cultures Technology, University of Zagreb Faculty of Food Technology and Biotechnology. The strains were stored as frozen stock at -80 °C in a CryoCube F101h ultra-low temperature freezer (Eppendorf, Hamburg, Germany) (Table 1), in de Man-Rogosa-Sharpe (MRS; Difco, Detroit, MI, USA) broth for lactobacilli, M17 broth (Biolife, Milan, Italy) for lactococci and enterococci and nutrient broth (Biolife, Milan, Italy) for test microorganisms, supplemented with 15 % (by volume) glycerol (Sigma-Aldrich, Saint Louis, MO, USA). Before every experimental trial, each strain was subcultured twice in a suitable growth medium under the listed growth conditions.

Antimicrobial activity

The antimicrobial activity of the LAB strains against test microorganisms and related LAB was determined using the turbidimetric method according to Leboš Pavunc *et al*. (*17*) with slight

modifications. The supernatant of overnight cultures of LAB strains isolated from human milk was filtered using sterile filter (Sigma-Aldrich, Saint Louis, MO, USA) with a diameter of 0.2 μm, and the filtrate was used in the experiment.

The antimicrobial activity of the selected LAB isolates from human milk was tested against test microorganisms (*S. aureus* ATCC®25925™, *L. monocytogenes* ATCC®19111™, *E. coli* ATCC®25922™ and *S. Typhimurium* FP1) and related LAB strains (*L. helveticus* M92, *L. lactis* subsp. *lactis* LMG 9450 and *E. faecium* ATCC®9430™) cultured in the appropriate medium until their A_{620} reached 0.5 and 1.0, respectively. 10 μL overnight cultures of the test microorganisms or related LAB strains were added to the wells of the microtitre plate (Greiner Bio-One, Kremsmünster, Austria) together with 90 μL of a suitable cultivation medium and 100 μL culture supernatants of selected LAB isolates from human milk. 10 μL of a culture of a specific test microorganism or a related LAB grown in 190 μL of the suitable cultivation medium was used as a control. The antibacterial activity of the culture supernatant was determined after 24 hours at 37 °C by spectrophotometric measurement of the apparent absorbance at a wavelength of 620 nm using a microtitre plate reader Infinite F Plex (Tecan, Männedorf, Switzerland).

Proteolytic activity

Determination of fast milk coagulation phenotype and acidification capacity

5 mL of the overnight cultures of the LAB strains was centrifuged at 8000×*g* for 10 minutes at 4 °C. The cells were washed twice with sterile phosphate-buffered saline (PBS, pH 7.4). 200 μL of each cell suspension was inoculated into 10 % (*m*/*V*) skimmed milk (Sigma-Aldrich, St. Louis, USA) and incubated at 37 °C for 16 h.

The ability of the LAB to coagulate milk was determined according to Hebert *et al*. (*18*). Depending on the rate of milk coagulation, the results were interpreted as follows: no coagulation, or as low, moderate, good or excellent coagulation.

Acidification capacity was measured in the supernatants by monitoring the pH change with a pH meter (Metrohm, Herisau, Switzerland) and by the titration method with 0.1 M NaOH (Carlo ERBA, Milan, Italy) with the addition of phenolphthalein indicator (Kemika, Zagreb, Croatia) until a pink colour appears. The acidity (°SH) was determined as follows:

$$°SH = V \cdot 20 \cdot f_{NaOH} \cdot 2 \qquad /1/$$

where V is the volume (in mL) of 0.1 M NaOH and f_{NaOH} is the correction factor of NaOH (1).

Proteinase plate assay

Mass per volume ratio of 10 % skim milk (Sigma-Aldrich, St. Louis, USA) was used to observe the proteinase phenotype of the strains according to Raveschot *et al*. (*19*) with slight modifications. Solid skim milk agar plates were routinely prepared by adding 1 % (*m*/*V*) agar (Biolife, Milan, Italy) to the medium. After solidification, sterile wells with a diameter of 7 mm were drilled and 50 µL of cell suspension or the supernatant of the overnight grown bacterial culture was added. Transparent halos were an indication of proteolytic activity.

Determination of proteolytic activity by Anson's method

The proteolytic activity of selected LAB strains was determined using the Anson method according to Beganović *et al*. (*16*). A volume of 1 mL of the supernatant filtrate of the overnight culture of each strain was suspended with 5 mL of a 0.65 % (*m*/*V*) casein solution in PBS (pH=7.2). After a 10-minute incubation at 37 °C, the reaction was halted by adding 5 mL of trichloroacetic acid (Thermo Fischer Scientific, Waltham, MA, USA), which led to precipitation of the non-hydrolysed proteins. After renewed incubation (30 min, 37 °C) and filtration, 5 mL of 0.4 M Na_2CO_3 solution (Kemika, Zagreb, Croatia) and 1 mL of Folin-Ciocalteau phenol reagent (Merck, Darmstadt, Germany) were added to 2 mL of filtrate. The sample was incubated again for 30 minutes at 37 °C and filtered. The absorbance was measured at 670 nm on a LKB 5060-006 microplate reader (LKB Vertriebs GmbH, Rome, Italy). The blank contained a pre-incubated casein solution to which trichloroacetic acid was added at the beginning of the experiment, followed by the addition of the supernatant and all the procedures described above.

Based on the linear equation, the amount (nmol) of L-tyrosine (Merck, Darmstadt, Germany) released was calculated by measuring the absorbance ($A_{670\ nm}$), which is due to the hydrolysis of casein by the action of proteases in the samples (Fig. 1).

Analysis of the casein degradation products by Tris-Tricine SDS-PAGE

The caseionolytic activity of selected bacterial strains was tested according to El-Ghaish *et al*. (*20*) with modifications. Overnight cultures were centrifuged at 3600×*g* for 10 min and then washed with phosphate buffer (pH=7.4). The resulting biomass was suspended in a 2 % (*m*/*V*) skimmed milk solution (Sigma-Aldrich, St. Louis, USA) and incubated at 37 °C for 48 hours. Casein degradation was then monitored using the tris-tricine sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) method. A non-inoculated skimmed milk solution was used as a control. The gel for Tris-Tricine SDS-PAGE was prepared according to Haider *et al*. (*21*).

Antioxidant activity

Overnight cultures of LAB strains were washed twice in PBS buffer (pH=7.4), after which the cells were mixed with freshly prepared DPPH (2,2-diphenyl-1-picrylhydrazyl hydrate) (0.2 mM in ethyl alcohol) in a 1:1 ratio and incubated in the dark for 30 minutes. After incubation, the samples were centrifuged and the absorbance of the supernatant was measured at 517 nm. Ethanol and PBS buffer (pH=7.4) were used as a blank test, and DPPH solution in ethyl alcohol and PBS buffer (pH=7.4) was used as a control. The ability to remove DPPH radicals was calculated according to the equation (*22*):

$$\% \text{ DPPH} = (1-(A_{\text{sample}}/A_{\text{control}}))\cdot 100 \qquad /2/$$

where DPPH is the percentage of 2,2-diphenyl-1-picrylhydrazyl hydrate, A_{sample} is the absorbance of the sample at 517 nm and A_{control} is the absorbance of the control at 517 nm.

Freeze-drying

Bacterial cells grown to late exponential phase in the optimal liquid nutrient medium were collected by centrifugation (3600×*g*), washed twice with sterile physiological solution and suspended in phosphate buffer (pH=7). The prepared suspension cells were then frozen overnight at -80 °C and freeze-dried for 24 h in Martin Christ Alpha 1–2 LDplus freeze dryer (Osterode, Germany).

Graphical representation and statistical analysis

The experiments were conducted in triplicate and the results are given as the mean of three independent experiments±standard deviation. All graphs, calculations and statistical analyses were made using GraphPad Prism v. 10.1.1 for Windows (*23)*. Ordinary one-way analysis of variance (ANOVA) was performed to calculate the significance of differences among multiple pairs of means in the data group. Differences between groups were considered significant when the p-value was below 0.05.

RESULTS AND DISCUSSION

Functional probiotic properties

Antibacterial activity

Isolation of LAB with desired functional properties from human sources is a tantalising approach to select potent probiotics for health-promoting applications (*24*). Selected LAB strains isolated from the human milk of Croatian women were previously identified by 16S RNA and whole genome sequencing, deposited in the NCBI database and characterised as producers of potential biotherapeutic molecules such as S-layer proteins (*L. brevis* MB1, MB2, MB13 and MB20),

exopolysaccharides (*L. fermentum* MC1) and plantaricins (*L. plantarum* KR19, MC19 and MB18) (*3*). These strains showed good survival under simulated gastrointestinal tract conditions, aggregation and adhesion to various epithelial and subepithelial structures of the intestinal tract (*11*). The aim of this study was therefore to assess the antimicrobial activity of selected LAB strains using the turbidimetric method in an appropriate liquid growth medium. The results were expressed as growth inhibition (%) of the tested bacteria compared to the control growth. The turbidimetric method was chosen due to the higher sensitivity compared to agar methods and due to the potential application of selected LAB strains isolated from human milk as functional starter cultures or even probiotics in fermented functional products. According to the results, after 24 h of incubation, the strains *L. plantarum* MC19 and *L. fermentum* MC1 showed the strongest ($p<0.05$) antimicrobial activity against the test microorganisms, while the antagonistic activity against the related LAB strains was depleted. *L. plantarum* MC19 strongly inhibited *S. aureus* ATCC®25925™, *S.* Typhimurium ATCC®14028™, *L. monocytogenes* ATCC®19111™, and *E. coli* ATCC®25922™ with rates of more than 80%. A similar antimicrobial activity was observed for *L. fermentum* MC1, with a significantly higher inhibition against *L. monocytogenes* ATCC®19111™ ($p=0.004$) compared to strain MC19 (Fig. 2a). A slightly lower, but still very high antimicrobial activity was observed for the strains *L. plantarum* MB18 and KR19 against both the test microorganisms and related LAB strains (Fig. 2b). In contrast, *L. brevis* MB13 showed no activity, while other S-layer expressing strains (*L. brevis* MB1, MB2, MB20) showed lower activity than other strains tested. The antimicrobial effect of LAB against common food-borne pathogens such as and *E*. coli, *L. monocytogenes* and *Salmonella* spp., results from the lowering of the pH of the medium due to the organic acids formed and the activity of synthesised bacteriocins, vitamins, EPSs and other metabolites with proven antimicrobial activity (*25,26*). While bacteriocins directly kill competing, closely related bacteria or pathogens or inhibit their growth by various mechanisms such as forming pores in the target cell membrane, disrupting ion gradients or inhibiting cell wall synthesis, S-layer proteins form a crystalline layer that covers a surface of the producing bacteria and contributes to structural integrity, mediates interactions with host tissue and modulates host immune responses (*11*). As a result, S-layers significantly enhance the adhesion of the producer to host cells and prevent pathogen adhesion without having direct bactericidal or bacteriostatic properties. This is probably the reason why S-layer producers have a less potent antimicrobial effect compared to bacteriocin producers. The *pln* loci of three investigated *L. plantarum* strains was disclosed by the detection of *pln*EF, *pln*A and *pln*J genes responsible for the production of the bacteriocins *Pln*EF, *Pln*A, and the peptide *Pln*J of the plantaricin *Pln*JK, using gene-specific primers (*3*). On the other hand, EPSs have health-promoting and rheological benefits in the food, pharmaceutical, and nutraceutical industries by exerting antimicrobial, antioxidant, immunomodulatory and many other biological functions. Strain *L.*

fermentum MC1 biosynthesises a mixture of three different polymers and harbours the genes involved in EPS production and transport, as well as a gene cluster related to bacteriocin production (*12*), which is a functional property contributing to antimicrobial activity. Strain MC1 also showed excellent adhesion properties, which are important for its probiotic activity. It can therefore be surmised that the strong antimicrobial activity of the plantaricin-producing *L. plantarum* strains and the EPS-producing *L. fermentum* MC1 is due to the biomolecules they produce.

Screening of proteolytic activity

The main component of human and bovine milk is β-casein, a protein that is a rich source of bioactive and antimicrobial peptides contributing to the endogenous peptidome of milk (*6*,*27*). Therefore, bovine skim milk was used as a milk model system for the evaluation of proteolytic capacity. Preliminary analyses of the selected autochthonous strains from human milk have shown that their ability to efficiently coagulate milk and exhibit a fast milk coagulation (Fmc) phenotype is a strain-dependent trait (Fig. 3a). *L. plantarum* MB15 showed a low coagulation efficiency, *L. brevis* MB20 a moderate one, the strains *L. plantarum* KR19, *L. plantarum* MB18, *L. brevis* MB13, *L. brevis* MB1 and *L. brevis* MB2 a good one, while the strain *L. fermentum MC1* showed an excellent coagulation efficiency. Determination of the fast milk acidification rate showed that the strains decreased the pH of the cultivation medium after overnight growth to values between (4.12±0.01) for *L. fermentum* MC1 and (3.81±0.08) for strain *L. plantarum* MB18, which is consistent with the degree of acidity (°SH) (Fig. 3b). Cervantes-Elizarrarás *et al*. (*28*) have reported that organic acids generated by LAB can suppress the growth of Gram-negative bacteria by penetrating their cell membranes and thus impairing their function, leading to acidification of the cytoplasm and inhibition of acid-sensitive enzymes. Although *L. fermentum* MC1 decreased the pH of the medium the least, it exhibited the strongest Fmc+ phenotype as determined by the fastest coagulation rate of the milk. This discrepancy can be explained by the fact that rapid milk coagulation does not necessarily require high acid production, as LAB can possess various traits and mechanisms, such as high enzymatic activity, that allow them to effectively coagulate milk proteins independently of acid production. Presumably, strain MC1 produces proteolytic enzymes that directly cleave milk proteins and cause effective coagulation without significantly lowering the pH of the medium.

Cell-envelope proteinases (CEPs) play a crucial role in the proteolytic system of LAB, as they are required to degrade proteins into peptides and/or amino acids, serving as a nitrogen source for LAB. Therefore, the presence of potential proteinase activity was tested in concentrated bacterial cell suspensions and in cell-free culture supernatants. The result showed that both the strains and their cell-free supernatants were able to hydrolyse the skim milk proteins as evident from the appearance

of the transparent halos around the wells in the agar, indicating casein hydrolysis (Fig. 3c). The mean diameter of the transparent halos was 10.83±1.24 for the LAB cell suspensions and 10.24±0.72 for the cell-free supernatants, indicating a slightly stronger caseinolytic activity of the LAB cells, albeit without significant meaning ($p>0.05$). However, the cell suspension of strain L. brevis MB20 showed significantly stronger ($p=0.036$) caseinolytic activity than its cell-free supernatant, suggesting that its proteolytic activity may be due to CEPs. These results are consistent with the research of Novak *et al*. (*6*), in which caseinolytic activity was detected in concentrated cell biomass of lactobacilli and lactococci strains isolated from various autochthonous fermented products.

Representative exopolysaccharide (*L. fermentum* MC1), bacteriocin (*L. plantarum* MB18) and S-protein (*L. brevis* MB1, *L. brevis* MB2) producers, whose cell-free culture supernatants and concentrated bacterial cell suspensions showed the highest proteinase activity, were further selected for evaluation of casein degradation potential. Human milk contains two classes of proteins, casein and whey proteins (*29*). In this study, the hydrolysis of casein and whey proteins into protein fragments and peptides was studied by Tris-Tricine SDS-PAGE (Fig. 4a). This resulted in the appearance of lower-intensity bands, implying that casein and whey proteins were partially degraded, i.e. a smaller amount of intact proteins remained. The strain *L. plantarum* MB18 showed the highest proteinase activity, which is consistent with the quantitative analysis of proteinase activities, where the amount of L-tyrosine released was (46.03±8.79) nmoL (Fig. 4b). According to the literature, many LAB belonging to the *L. plantarum* strains produce peptides with numerous bioactive effects such as anti-inflammatory, antihaemolytic, antioxidant, antimutagenic or antimicrobial effects through the fermentation of milk (*30*). Since infant formulas are highly rich in casein, which makes them difficult to digest compared to human milk, supplementation with strains expressing active proteinase could eventually contribute to improved casein digestibility (*31*). This feature is attractive from various aspects of the application of lactobacilli strains, whether as a probiotic supplement in cow's milk-based infant formula or as a starter culture in fermented food products as this can lead to the accumulation of health-promoting bioactive peptides.

Antioxidant activity

An imbalance in the body can be caused by oxidative stress, which leads to damage to cells and tissues, triggered by the excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). As a result, various diseases can develop, such as diabetes, cancer, cardiovascular problems and inflammatory and neurological diseases. For this reason, it is necessary to develop supplements with antioxidant effect in order to reduce oxidative stress, and here the potential of probiotics has also gained tremendous scientific importance (*13*,*32*). During food

fermentation, the antioxidant activity of LAB can be attributed to bioactive peptides, EPSs, organic acids and a change in the pH of the environment, which can lead to an increase in their bioavailability (*5*,*33*).

Therefore, the antioxidant activity of the selected strains was evaluated by DPPH radical scavenging activity. Strain *L. brevis* MB2 showed the highest and strain *L. plantarum* KR19 the lowest radical scavenging activity, while strains MB1, MB13, MB20, MB18, MC1 and MC19 showed similar DPPH radical scavenging activity of about 50 % (Fig. 5). Using the same method, Vougiouklaki *et al*. (*34*) reported that *Lactobacillus gasseri* ATCC 33323 removed 78 % of DPPH radicals, while the values for *Lacticaseibacillus rhamnosus* GG ATCC 53103, *Levilactobacillus brevis* ATCC 8287 and *Lactiplantibacillus plantarum* ATCC 14917 were between 33 and 39 %.

Among all the strains tested, the EPS-producing *L. fermentum* MC1 and the plantaricin-producing *L. plantarum* MB18 strains can find potential application due to their desirable properties. Proteolytic activity is a prerequisite for starter culture application, which can result in production of bioactive peptides with numerous functional properties (*5*). Due to their ability to inhibit the growth of a wide spectrum of bacteria, they can serve as biopreservatives that can extend the shelf life of the consumed product. In addition to antimicrobial properties, antioxidant activity may also be important in extending the shelf life of functional products as well as for protection from oxidative damage in the human body after consumption, along with their functional role, while EPS production can have a positive impact on rheological properties. Overall, all these effects can additionally improve the nutritional relevance of functional products with functional roles for host health, such as gut microbiota balance.

Technological probiotic properties

Drying processes are often used to stabilise probiotic ingredients by reducing their moisture content and facilitating their transport and preservation, with freeze-drying being the most commonly used method. The probiotic powder acquired by freeze-drying can successfully maintain the viability of probiotics and has a satisfactory fermentation performance (*35*). Although cryoprotectants are often used to support the survivability of bacteria, we used only phosphate buffer to investigate the ability of LAB strains isolated from human milk to survive the extreme conditions during freeze-drying. According to the results (Table 2), the most remarkable survival rate after freeze-drying was observed in *L. brevis* MB1 and MB20 strains, with a loss of only (1.086±0.015) and (1.003±0.047) logCFU/mL, respectively. On the other hand, *L. plantarum* strains showed a greater loss of viable cells, especially strain *L. plantarum* MC19 with a loss of (4.53±0.11) logCFU/mL, while *L. plantarum* MB18 lost only (1.85±0.01) logCFU/mL. This phenomenon may be due to the expression of S-layer proteins on the

cell surface of the *L. brevis* strains, which also showed a protective role in simulated gastrointestinal passage and increased adhesion to the Caco-2 cell line (*10*). All strains, with the exception of *L. plantarum* MC19, excreted more than 10^6 CFU/mL after freeze-drying, which is a generally recognised requirement for probiotics to have a therapeutic effect at the time of consumption (*36*). Overall, all tested strains, with the exception of *L. plantarum* MC19, fulfil the primary technological criterion for selecting probiotic strains.

CONCLUSIONS

Our data provide interesting insights into the specific probiotic features and potential use of LAB isolated from the human milk microbiota in functional products, especially the EPS-producing strain *L. fermentum* MC1 and the plantaricin-producing *L. plantarum* MB18. The beneficial properties of these cultures, exerted through functional, technological and safety criteria, may be useful for the production of fermented products with added functional value and with potential nutritional and functional relevance for the host. Their potential application may be directed towards their use as bio-preservatives to reduce the use of chemical additives, which meets consumer demand for more natural and environmentally friendly products.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

K. Butorac performed the analysis of proteolytic and antioxidant activity, draft the article, designed the work, performed the graphical presentation, data analysis and interpretation. M. Banić collected and identified the lactic acid bacteria strains, performed the analysis of antimicrobial activity and freeze-drying, draft the article, data analysis and interpretation. B. Kos supervised and critically revised the manuscript and finally approved the version to be published. N. Čuljak participated in

statistical analysis of the results and preparation of the manuscript. D. El Khalifa performed the analysis of antimicrobial and antioxidant activity. E. Habuš performed the analysis of proteolytic activity. A. Leboš Pavunc and J. Novak were involved in the preparation and critical revision of the manuscript. J. Šušković approved the final version to be published. All authors approved the final version of the manuscript for publication.

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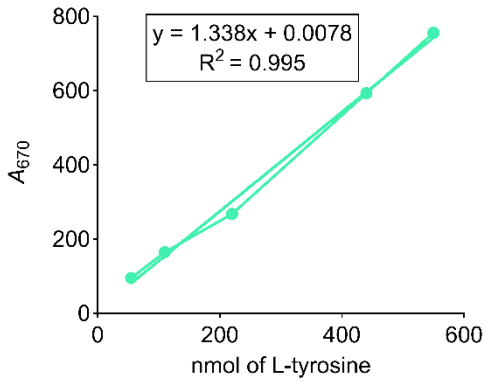

Fig. 1. Standard curve for the measurement of L-tyrosine and corresponding linear equation for the determination of proteolytic activity by Anson's method

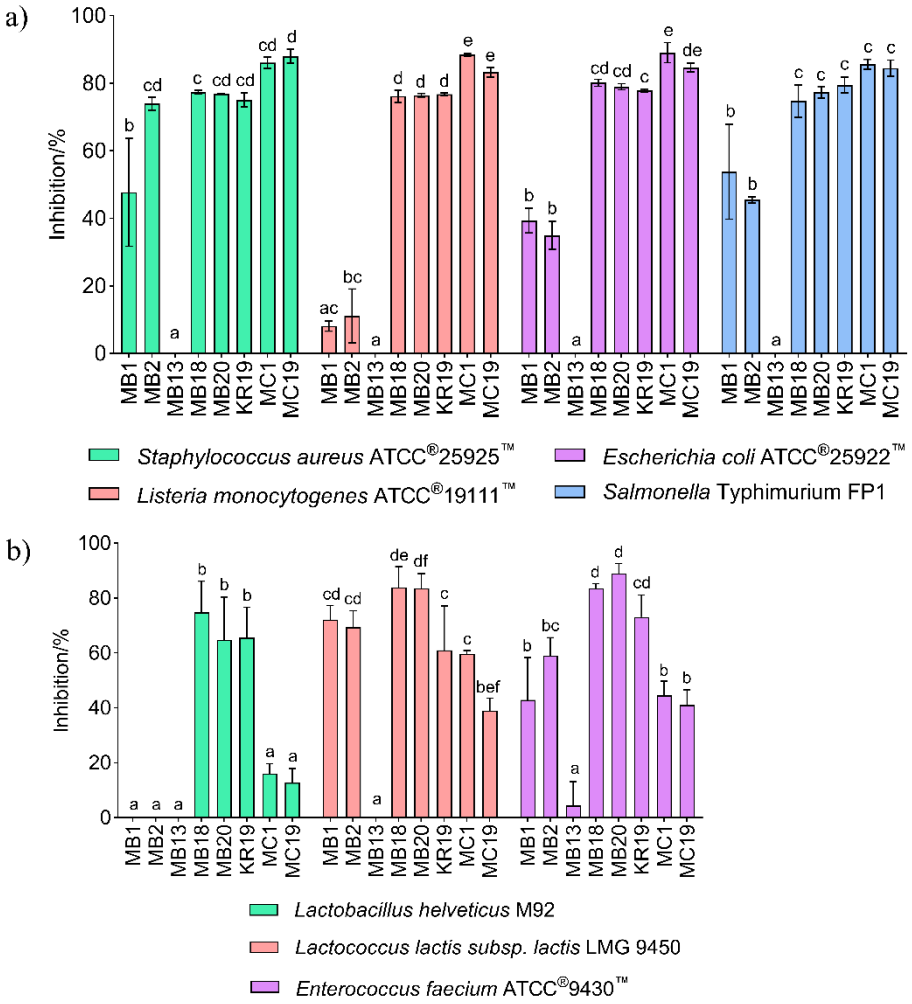

Fig. 2. Antimicrobial activity of LAB strains against: a) test microorganisms and b) related LAB strains tested by the turbidimetric method. Different letters in superscript indicate statistically significant difference ($p<0.05$) between the LAB strains against the same test microorganism *i.e.* a related LAB strain

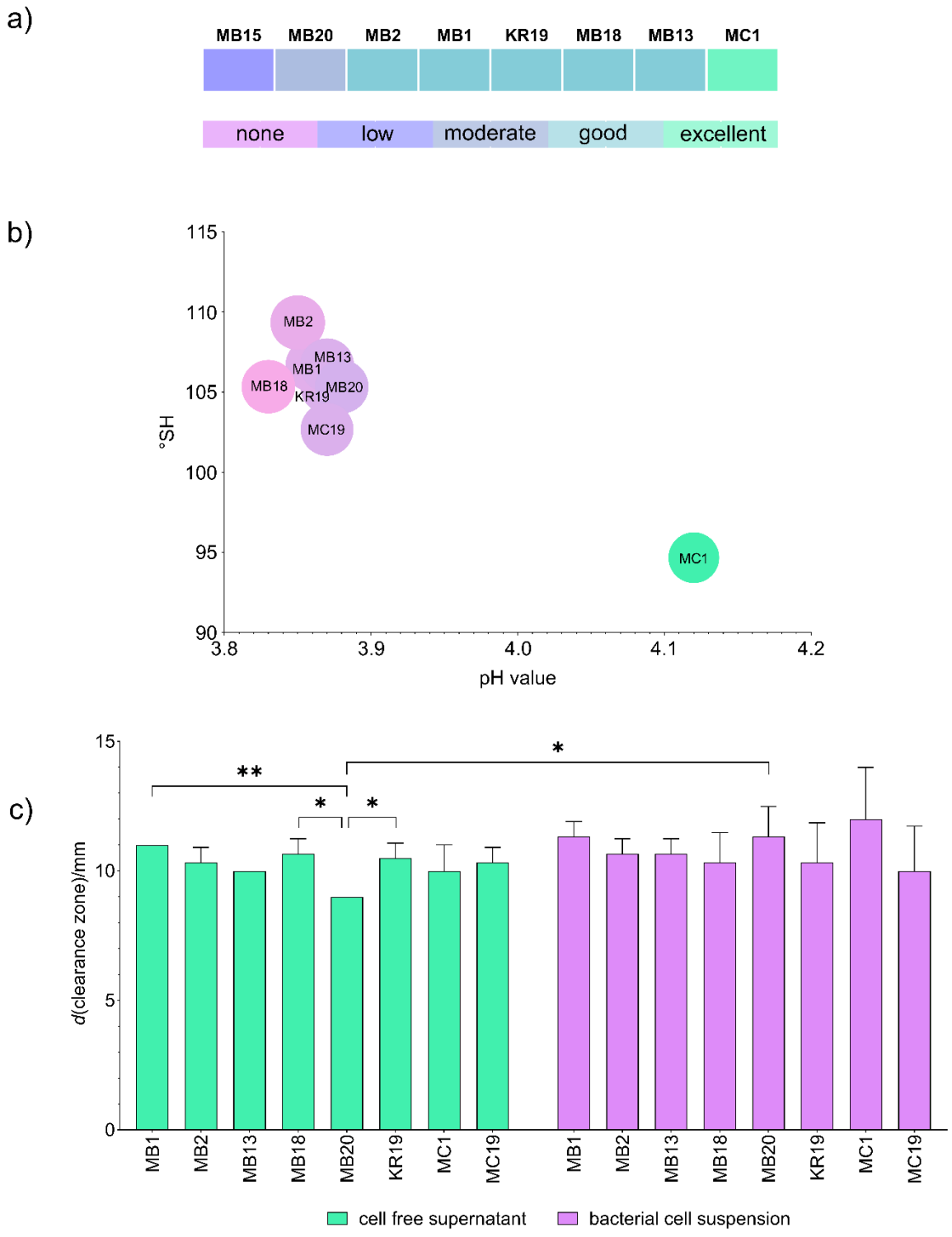

Fig. 3. a) Milk coagulation efficiency, b) pH values and degree of acidity (°SH) after overnight incubation and c) proteinase activity of cell-free culture supernatants and concentrated bacterial cell

suspensions of *Lactobacillus* strains isolated from human milk. Asterisks indicate significant differences at different levels: *p<0.05, **p<0.01

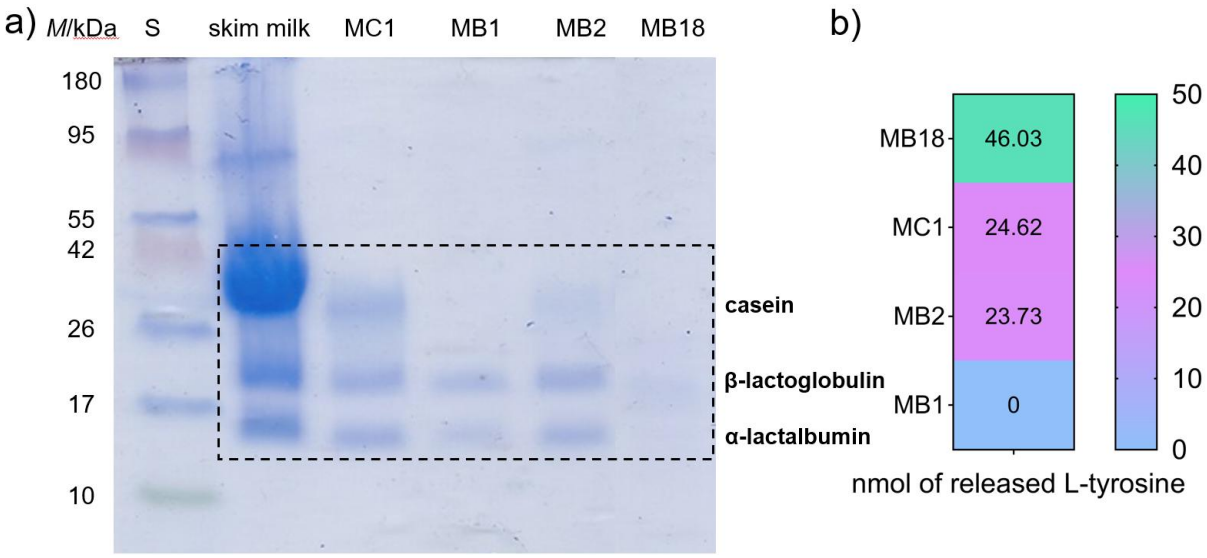

Fig. 4. a) Tris-Tricine SDS-PAGE analysis of samples obtained after hydrolysis of skim milk and b) amount (nmol) of L-tyrosine released by potential proteolytic activities of selected LAB strains determined by the Anson's method

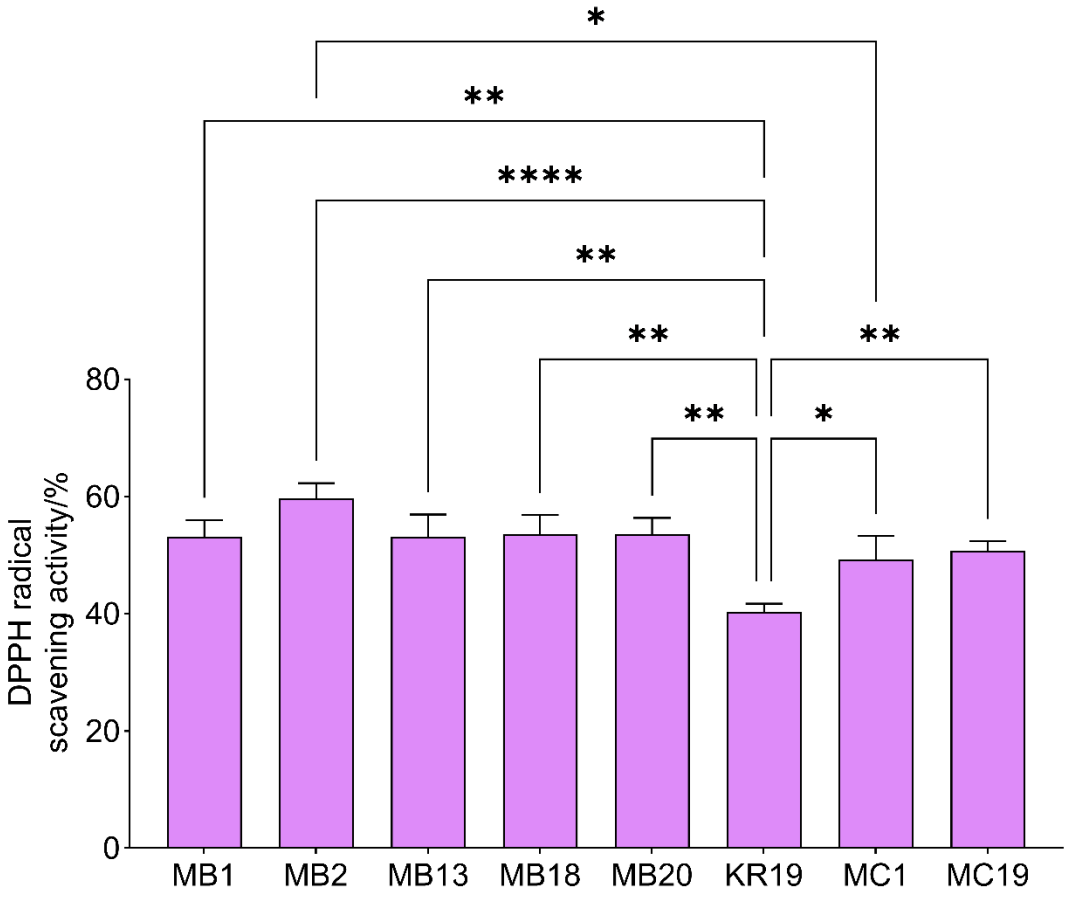

Fig. 5. Antioxidant activity as % of DPPH scavenging of selected *Lactobacillus* strains. Asterisks indicate significant differences at different levels: *p<0.05, **p<0.01, ****p<0.0001

Table 1. Bacterial strains used in this study and their cultivation conditions

Bacterial strain	Cultivation	Origin	Bioactive molecule
Lactiplantibacillus plantarum KR19	MRS, 37 °C, microaerophilic*	human milk	bacteriocin (*3*)
Limosilactobacillus fermentum MC1	MRS, 37 °C, microaerophilic*	human milk	exopolysaccharide (*3*)
Lactiplantibacillus plantarum MC19	MRS, 37 °C, microaerophilic*	human milk	bacteriocin (*3*)
Levilactobacillus brevis MB1	MRS, 37 °C, microaerophilic*	human milk	S-protein (*3*)
Levilactobacillus brevis MB2	MRS, 37 °C, microaerophilic*	human milk	S-protein (*3*)
Levilactobacillus brevis MB13	MRS, 37 °C, microaerophilic*	human milk	S-protein (*3*)
Lactiplantibacillus plantarum MB18	MRS, 37 °C, microaerophilic*	human milk	bacteriocin (*3*)
Levilactobacillus brevis MB20	MRS, 37 °C, microaerophilic*	human milk	S-protein (*3*)
Lactobacillus helveticus M92	MRS, 37 °C, microaerophilic*	fermented milk	S-protein (*16*)
Lactococcus lactis subsp. *lactis* LMG 9450	M17, 30 °C, aerobic	BCCM	/
Enterococcus faecium ATCC®9430™	M17, 37 °C, aerobic	ATCC	/
Staphylococcus aureus ATCC®25925™	nutrient broth, 37 °C, aerobic	ATCC	/
Listeria monocytogenes ATCC®19111™	nutrient broth, 37 °C, aerobic	ATCC	/
Escherichia coli ATCC®25922™	nutrient broth, 37 °C, aerobic	ATCC	/
Salmonella enterica serovar Typhimurium ATCC®14028™	nutrient broth, 37 °C, aerobic	ATCC	/

*Microaerophilic conditions were created using Anaerocult A system (Merck, Darmstadt, Germany). BCCM=Belgian Coordinated Collections of Microogranisms, ATCC=American Type Culture Collection

Table 2. The number of viable cell count of LAB strains isolated from human milk before and after freeze-drying in phosphate buffer

LAB strain	Before freeze-drying *N*/(logCFU/mL)	After freeze-drying *N*/(logCFU/mL)	Freeze drying survival rate/%
L. plantarum KR19	(9.722±0.003)^{a}	(6.951±0.033)^{b}	(71.498±0.033)
L. fermentum MC1	(9.957±0.074)^{a}	(7.847±0.083)^{b}	(78.809±0.111)
L. plantarum MC19	(9.656±0.019)^{a}	(5.122±0.109)^{b}	(53.045±0.111)
L. brevis MB1	(9.245±0.004)^{a}	(8.159±0.017)^{b}	(88.253±0.017)
L. brevis MB2	(10.228±0.003)^{a}	(8.272±0.015)^{b}	(80.876±0.015)
L. brevis MB13	(9.671±0.017)^{a}	(7.995±0.069)^{b}	(82.670±0.071)
L. plantarum MB18	(8.855±0.011)^{a}	(7.003±0.005)^{b}	(79.085±0.012)
L. brevis MB20	(9.017±0.021)^{a}	(8.014±0.042)^{b}	(88.876±0.047)

Results are reported as mean value±standard deviation. Different letters in superscript indicate statistically significant difference ($p<0.05$) between the same strain before and after freeze-drying