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Korrespondenzadresse:
meltemozusaglam@gmail.com

[1]) Department of Molecular Biology and Genetic, Faculty of Science and Letters, Aksaray University, 68100, Aksaray, Turkey; [2]) Institute of Biotechnology, Ankara University, Ankara, 06110, Turkey

Investigation of a new *Lactobacillus delbrueckii* strain from human milk as a probiotic candidate

Untersuchung eines neuen Lactobacillus delbrueckii-Stammes aus menschlicher Milch als probiotischer Kandidat

Meltem Asan-Ozusaglam[1]), Ayse Gunyakti[1,2])

Summary

In this study, the safety evaluation, health-beneficial potential, probiotic and functional properties of *Lactobacillus delbrueckii* MA-9 strain originating from human breast milk were investigated in vitro. MA-9 strain showed very high resistance to simulated in vitro gastrointestinal conditions and food preservatives. The strain demonstrated non-hemolytic activity, inhibitory action on different indicator pathogen microorganisms (twelve clinical or human food-borne bacteria, two yeast and seven bacterial fish pathogens), high levels of aggregation, strong cholesterol removal ability and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (43.40%). Moreover, the strain showed positive α-amylase and lichenase (ß-1,3-1,4-glucanase) enzyme activities. The results demonstrated that the *L. delbrueckii* MA-9 strain can be a good probiotic candidate and natural food additive to promote health.

Keywords: Human milk, enzyme activity, anti-cholesterol ability, aggregation

Introduction

The upper respiratory system, gastrointestinal tract and urogenital system of all vertebrate organisms, including humans, have a very large microbial population. The majority of these microbial populations are bacteria and these are most commonly localized in the intestinal and immune system (10^{10}–10^{12} CFU/g) (Kerry et al., 2018; Parker et al., 2020). The beneficial-harmful microorganisms in the gastrointestinal tract of a healthy host are in equilibrium and the metabolites of useful microorganisms have many beneficial/positive effects on host health. For example, probiotic microorganisms can support the maintenance and repair of the epithelial barrier by producing various metabolic byproducts (such as polysaccharides, short-chain fatty acids) that induce mucus secretion of intestinal cells and the synthesis of tight junction proteins (Silva et al., 2020; Song et al., 2021). However, metabolite output of the intestinal microflora can influence not only intestinal health but also the function of many other distal organs and systems through some axis such as; gut-brain, gut-lung, gut-liver, gut-kidney, gut-bone, gut-muscle (Schroeder and Bäckhed, 2016; Cianci et al., 2018; Feng et al., 2018; Grosicki et al., 2018; Yang et al., 2018). In the case of impaired gut microbiota (dysbiosis), there is a decrease in the number-diversity of the beneficial microorganisms and their metabolites. In dysbiosis, the intestinal epithelial cells are destroyed and consequently, the flow of undigested food and toxic substances into the bloodstream takes place (Parker et al., 2020). In such a case, re-modulation of health and the regulation of intestinal-associated multiple host metabolic pathways can be achieved by consuming probiotic-supplemented foods.

Probiotics have been currently defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (Hill et al., 2014). It has been determined that probiotic microorganisms have important therapeutic effects on host health such as feeding other intestinal microbial communities, providing energy homeostasis and affecting the functions of various organs and systems (Wang et al., 2017). Most of the strains identified as probiotic cultures are found in the lactic acid bacteria (LAB) group and these microorganisms are widely used in the fields of medicine, food and feed production.

Lactobacillus delbrueckii group members are obligately homofermentative lactobacilli and the G + C ratio in their DNA is in the range of 49–51% mole (Rizzello and De Angelis, 2011; Hammes and Hertel, 2006). *L. delbrueckii* strain has been genetically identified in many studies in the past to determine the bacterial diversity of breast milk (Collado et al., 2009; Xu et al., 2018; Ding et al., 2019). However, studies focusing on the determination of probiotic properties mostly cover *L. delbrueckii* subsp. *bulgaricus* strains from yogurt and fermented food (Nwamaioha and Ibrahim, 2018). Studies on *L. delbrueckii* strains of human milk origin are very limited and it is always desirable to identify new strains with probiotic potential. Because generally, the probiotic properties of these microorganisms are specific for strain. Therefore, in this study, susceptibility of *L. delbrueckii* MA-9 strain to the gastrointestinal system, food preservatives and various antibiotics was tested in an *in vitro* experiment. In addition, some properties that can modulate health such as antimicrobial, antioxidant and anti-cholesterol activities and aggregation abilities have been investigated.

Material and methods

Strain

L. delbrueckii MA-9 was isolated from breast milk collected from volunteer mothers in Aksaray University Scientific and Technological Application and Research Center (ASUBTAM) as part of a project we have previously carried out.

2.2. Safety screening

The susceptibility of MA-9 strain to antibiotics was performed using the disc diffusion method on MRS medium (Anisimova and Yarullina, 2019). Ten commercial antibiotic discs (Oxoid) were used in the study. The antibiotic discs used and their concentrations were as follows: amikacin, (10 µg), gentamycin (10 µg), kanamycin (30 µg), amoxicillin (30 µg), ampicillin (10 µg), penicillin G (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), nalidixic acid (30 µg), ofloxacin (5 µg). The active culture of *L. delbrueckii* MA-9 strain (McFarland 0.5 = 5.1×10^8 CFU/mL, 100 µl) was spread on MRS agar plates. The antibiotic discs were placed on the surface of the inoculated plates. Petri dishes were placed in an anaerobic jar containing Anaerocult® (Merck) moistened with approximately 5 mL of distilled water and incubated at 37°C for 24 h. The inhibition zone diameters were measured and the results were presented as resistance (R), moderate susceptibility (MS), or susceptibility (S) according to interpretative standards described previously (Charteris et al., 1998).

Screening for hemolytic activity was performed using Columbia sheep blood agar (Çağdaş 06 Sağlık Hizmetleri Ltd.Şti, OR-BAK, Lot number: 1602472). Briefly, streak plate method was used for inoculation of MA-9 strain on the growth media surface. After incubation at 37°C for 24 h, the plates were examined for the formation of blood lysis zones (α, ß or γ-hemolysis) around MA-9 colonies.

Resistance to Simulated Gastrointestinal Conditions

Tolerance to simulated gastrointestinal conditions (low pH, bile, gastric and small intestinal fluids) was tested by viable colony count. For this purpose, MRS broth and agar medium were prepared separately to simulate the different conditions mentioned. In order to detect the low pH resistance of the strain, media with pH 2 and 3 were prepared, while media containing 0.3% and 1.0% (Thermo Scientific™ Oxoid™ Bile) bile were prepared to determine their viability at different bile concentrations (Reuben et al., 2019). In order to determine the simulated gastric juice tolerance of the strain, media containing 3 g/L (Sigma-Aldrich) pepsin adjusted to pH 2 and 3 were prepared. Finally, MRS broth and agar media containing 1 g/L pancreatin (Sigma-Aldrich)-0.03 g/L bile salt (Oxoid) were prepared to determine the tolerance of the strain to the small intestinal fluid (Asan-Ozusaglam and Gunyakti, 2019; Kaur et al., 2020). Free cell concentrations of *L. delbrueckii* MA-9 strains were adjusted based on the Mc Farland 0.5 (≅5.1×10^8 cfu/mL) system in saline solution (Bin Masalam et al., 2018; Sharafi and Nateghi, 2020). Concentration-adjusted cell suspension, sterile control group and test group were inoculated separately at 1% in MRS broth. After inoculation, control and test groups were incubated for 0, 1 and 3 hours for low pH tolerance, 0 and 4 hours for bile tolerance, 0 and 3 hours for stomach and 0 and 4 hours for small intestine solutions. These incubation times used in the experiments were chosen based on the exposure times of the foods to the gastrointestinal tract. At the end of the

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incubation periods, serial dilutions were made in SF solutions for all groups. From the prepared dilution sets, 1% inoculation was performed on MRS agar media and petri plates were incubated at 37°C for 24 hours under anaerobic conditions. At the end of the incubation period, viable cell counts were performed and the obtained values were presented based on Log10. The experiments were performed with three replicates.

Determination of Aggregation Abilities

The aggregation assays were assayed according to Behbahani et al. (2019) with slight modifications. The co-aggregation ability of *L. delbrueckii* MA-9 strain with various pathogen microorganisms such as *L. monocytogenes* ATCC 7644, *E. coli* ATCC 35218, *E. coli* O157:H7 ATCC 43895, *S. enteritidis* ATCC 13076, *S. enteritidis* RSKK 171, *S. agalactiae* and *V. alginolyticus* was tested. For both experiments, the cell densities of MA-9 strain and pathogenic test bacteria were adjusted to a density of 0.6 ± 0.02 at 600 nm (≅12x 10^8 CFU/mL) with a spectrophotometer (Beckman Coulter DU 730) in phosphate-buffered saline (PBS). For the auto-aggregation assay, *L. delbrueckii* MA-9 strain was incubated at 37 °C for 4 h without agitation in three replicates. For the co-aggregation assay, equal volumes (2 mL) aliquots of *L. delbrueckii* MA-9 strain and pathogenic microorganisms were mixed and then incubated at 37 °C for 4 h. After the incubation period, the sample (0.1 mL) was suspended in PBS buffer (3.9 mL) and read at OD_{600} nm for auto-aggregation and co-aggregation assays.

The percentage of auto-aggregation was expressed as follows:

$$\text{Auto-aggregation \%} = \frac{OD1 - OD2}{OD1} \times 100$$

OD1: pre-incubation absorbance, OD2: after incubation absorbance

The percentage of co-aggregation was calculated as follows:

$$\text{Co-aggregation \%} = \frac{(OD\ strain + OD\ pathogen\ right) - 2(OD\ mix)}{(OD\ strain + OD\ pathogen)} \times 100$$

ODstrain: absorbance of *L. delbrueckii* MA-9 (pre-incubation), ODpathogen: absorbance of pathogen strain (pre-incubation), ODmix: absorbance of mixed strains (after 4 h)

Determination of tolerance to food preservative substances

The tolerance of *L. delbrueckii* MA-9 to sodium benzoate (Sigma Aldrich Saint Louis, USA, CAS number: 532-32-1) and nisin (Sigma Aldrich Saint Louis, USA, CAS number: 1414-45-5) which are used as food preservatives was investigated. The sodium benzoate resistance of the strain (McFarland 0.5≅10^8 CFU/mL) was tested spectrophotometrically (OD_{600} nm) in MRS broth medium prepared in seven different concentrations (0.015–1%) and acid production capabilities were determined at the same concentrations. The viability of the strain was also assayed at 0.1% sodium benzoate on solid agar after 24 h incubation at 37°C. The tolerance of the strain to various concentrations of nisin (1.25-150 µg/mL) was tested using the well diffusion method (Tsai and Sandine, 1987). The inhibition zones were recorded after incubation at 37 °C for 24 h.

Determination of Enzymatic Activities

Amylase and lichenase (ß-1,3-1,4-glucanase) enzyme activities of *L. delbrueckii* MA-9 were qualitatively determined by spot-dropping free-cell supernatants on agar media. Nutrient agar media containing soluble starch (0.5% w/v) was used for amylase enzyme activity while medium containing Lichenan (0.1% w/v) was used for lichenase enzyme activity. The free cell supernatant (50 µL) was dropped onto agar and incubated for 30 min at 37 °C. Then, the plates were stained with iodine and Congo Red to determine amylase and lichenase enzyme activities. The forming of clear zone around the spot-dropping areas on the agar medium was considered positive for the enzyme activity (Aşan and Ozcan, 2007).

Antimicrobial Activity

The antimicrobial effect of sterile cell-free supernatant (CFS) of 18–24 hours of active *L. delbreuckii* MA-9 culture against the fourteen clinical human-food borne bacteria, two yeast and seven bacterial fish pathogens were investigated using the agar well diffusion method described by Prabhurajeshwar and Chandrakanth (2017) with slight modifications. Active MA-9 culture was centrifuged at 5000 rpm for 15 minutes and bacterial cell-free supernatant (CFS) was separated from the pellets. CFS was filtered with the help of 0.2 µm micro filters, taken into sterile glass tubes and stored at +4°C for use in the experiment. The indicator strains (Mc Farland 0.5 standard turbidity, 100 µL) were inoculated to the appropriate agar media. The sterile CFS (100 µL) was placed into the wells (7 mm in diameter). Following inoculation, the plates were incubated at the appropriate temperature for the development of indicator microorganisms for 24 h. Suitable media and incubation temperatures used for culturing pathogenic strains are as follows; Tryptic Soy Broth (TSB) medium containing 2% NaCl and 25°C for Vibrio species, TSB medium/25°C for *L. garvieae, Y. ruckeri* strains, TSB medium/37 °C for *S. agalactiae* strain, Nutrient medium/30°C for *A. hydrophila* ATCC 1970 strain, Yeast Extract-Peptone-Dextrose (YPD) medium/30°C for Candida species, TSB medium/37°C for *E. faecalis* ATCC 29212, *L. monocytogenes* ATCC 7644 strains, and Nutrient medium/37°C for all other indicator pathogen microorganisms. Antimicrobial activity experiments were performed in triplicate and at the end of the incubation period the inhibition zone diameter values were measured using automatic caliper. The results were given as mean ± standard deviation.

Antioxidant Activity

Antioxidant activity of *L. delbreuckii* MA-9 strain was performed by DPPH (2,2-diphenyl-1-picrylhydrazyl; Sigma-Aldrich, Cas no:1898-66-4) radical scavenging and ferrous-ion chelating activity methods according to Chenet al. (2014) and Parrella et al. (2012) with minor modifications. For the DPPH radical reduction activity, 0.2 mM methanolic DPPH solution and of MA-9 strain (Mc Farland 0.5 standard turbidity, 0.8 mL) were mixed and then kept in the dark at room temperature for 30 min. After the incubation period, the absorbance of the mixed solution was read with the spectrophotometer at OD_{517} nm. For ferrous-ion chelating activity (FCA), ferrozine solution (5 mM, 2 mL), $FeCl_2$ (2 mM, 0.05 mL) solution and MA-9 strain (Mc Farland 0.5 standard turbidity, 1 mL) were mixed and then the absorbance was measured by using the spectrophotometer at OD_{562} nm. The DPPH radical scavenging and

ferrous ion chelating activities were estimated using the following formulas:

$$\text{DPPH radical scavenging activity (\%)} = 1 - \frac{OD2}{OD1} \times 100$$

$$\text{Ferrous ion chelating activity (\%)} = 1 - \frac{OD1 - OD2}{OD1} \times 100$$

OD1= the absorbance of the control and OD2= the absorbance of the sample.

Determination of Cholesterol Assimilation Ability

The cholesterol assimilation ability of *L. delbrueckii* MA-9 strain was determined by using the „o-phthalaldehyde" descripted assay as we previously reported (Gunyakti and Asan-Ozusaglam, 2018). Briefly, strain MA-9 in MRS broth containing 1% Cholesterol (Sigma-Aldrich, Saint Louis, USA) (100 µg/mL) and bile (0.3% vs 1%) (Oxoid, Hampshire, UK) were inoculated and incubated at 37 °C for 24 hours. At the end of the incubation period, the strain was centrifuged to obtain supernatant and pellets. The pellets were suspended in distilled water equal volume to the previous original media. Then, 3 mL of 99% ethanol was added into 0.5 mL of supernatant or pellet and vortex-mixed. 2 mL of 50% KOH (Sigma-Aldrich, Saint Louis, USA) was added to the samples and heated in a water bath (60 °C) for 10 min and then cooled at room temperature. 5 mL of hexane was added to the samples and the mixture was stirred for 20 s. Distilled water (3 mL) was added to each sample and gently mixed. The samples were kept at room temperature for 15 min. 2.5 mL of the hexane layer was taken and completely evaporated in a water bath at 60 °C. 4 mL of o-phthalaldehyde reagent (Sigma-Aldrich, Saint Louis, USA) was added to each tube. After 10 min, 2 mL of pure sulfuric acid (Merck, Darmstadt, Germany) was added to the samples. The samples were mixed and then incubated at room temperature for 10 min. Absorbance was read using a spectrophotometer at 550 nm. Cholesterol assimilation was calculated using the equation:

$$A = 100 - [(B/C) \times 100]$$

where A is cholesterol assimilation (%), B is cholesterol content in the culture medium(µg), C is cholesterol content in the non-inoculated (control) medium (µg).

Result and discussion

Safety aspects

The hemolytic and antibiotic resistance activities of the *L. delbrueckii* MA-9 strain were determined for the in vitro safety assessment. The resistance of MA-9 strain to various group antibiotics was evaluated and inhibition zone diameter values around antibiotic discs are given in millimeter (Table 1). Inhibition zone values obtained as a result of the antibiogram test were determined in the range of 7.44–29.61 mm. It was determined that the MA-9 strain was resistant (Resistant≤14) to all aminoglycoside (amikacin, gentamicin, kanamycin) and Quinolone (nalidixic acid, ofloxacin) group antibiotics. Conversely, the strain showed sensitivity against Beta-lactam (amoxicillin, ampicillin, penicillin G), Chloramphenicol (chloramphenicol) and Macrolide (erythromycin) groups (Susceptible>20). *L. delbrueckii* MA-9 strain showed the highest sensitivity with an inhibition zone value of 29.61 mm against penicillin G.

The resistance to antibiotic of probiotic microorganisms is regarded as a safety concern due to the risk of gene transfer. However, it has been reported that if a strain shows intrinsic resistance to any antibiotic, these resistance traits are not transferrable by horizontal gene transfer to other microorganisms in the intestinal microbiota (de Melo Pereira et al., 2018). In the literature, it has been determined that some Lactobacillus species such as *L. delbrueckii, L. rhamnosus, L. helveticus* and, *L. acidophilus* have intrinsic resistance to aminoglycoside and quinolone group antibiotics (Hummelet al., 2007; Sharma et al., 2017; Tang et al., 2018; Botthoulath et al., 2018). Considering the literature findings, a similar antibiotic resistance pattern was obtained for the *L. delbrueckii* MA-9 strain in our study. This may indicate that the MA-9 strain has intrinsic/innate resistance to aminoglycoside and quinolone group antibiotics, but further confirmation is still needed. The ability of the MA-9 strain to resist aminoglycoside and quinolone group antibiotics may be beneficial for the restoration of intestinal microflora destroyed during or after antibiotic treatment. One of the important selection criteria is a hemolytic activity for probiotic microorganisms. Lactic acid bacteria showing alpha-hemolytic activity were evaluated as non-hemolytic in the previous literature reports (Aryantiniet al., 2017; García et al., 2017; Peres et al., 2014). Similarly, in this study, it was determined that *L. delbrueckii* MA-9 showed α-hemolytic activity and based on this result, it was a non-hemolytic strain. On the basis of these results, it is considered that the MA-9 strain does not carry any risk factors for host health.

Cell survival in simulated gastrointestinal conditions

The tolerance of *L. delbrueckii* MA-9 to the simulated gastrointestinal tract conditions was assayed by counting viable cell and the results are given in $\log_{10}$ CFU/mL (Figure 1). In the viability of the strain, an increase was observed in the first hour of incubation in culture medium at

TABLE 1: *Antibiotic susceptibility of L. delbrueckii MA-9 strain.*

Aminoglycoside group	Antimicrobial susceptibility	Mean ± standard deviation	Break point of susceptibility[c] R	MS	S
AK (10 µg)	R	–[a]	≤15	16–17	≥18
CN (10 µg)	R	7.44±0.49	≤12	–	≥13
K (30 µg)	R	–	≤13	14–17	≥18
Beta-lactam group					
AMC (30 µg)	S	24.4±0.80[c]	≤18	19–20	≥21
AM (10 µg)	S	23.73±0.75	≤12	13–15	≥16
P (10 µg)	S	29.61±2.60	≤19	20–27	≥28
Chloramphenicol group					
C (30 µg)	S	20.76±1.07	≤13	14–17	≥18
Macrolide group					
E (15 µg)	S	28.64±0.79	≤13	14–17	≥18
Quinolone group					
NA (30 µg)	R	–	≤13	14–17	≥18
OFX (5 µg)	R	–	≤13	14–18	≥19

[a]: Indicates no inhibition zone. [b]: Diameter of the inhibition zone including disc diameter. Values are reported as means ± SD of three separate replicates. **AK:** Amikacin, **AMC:** Amoxicillin, **AM:** Ampicillin, **C:** Chloramphenicol, **E:** Erythromycin, **CN:** Gentamycin, **K:** Kanamycin, **NA:** Nalidixic Acid, **OFX:** Ofloxacin, P: Penicillin G, [c]: R (resistant), MS (moderately susceptible), S (susceptible)

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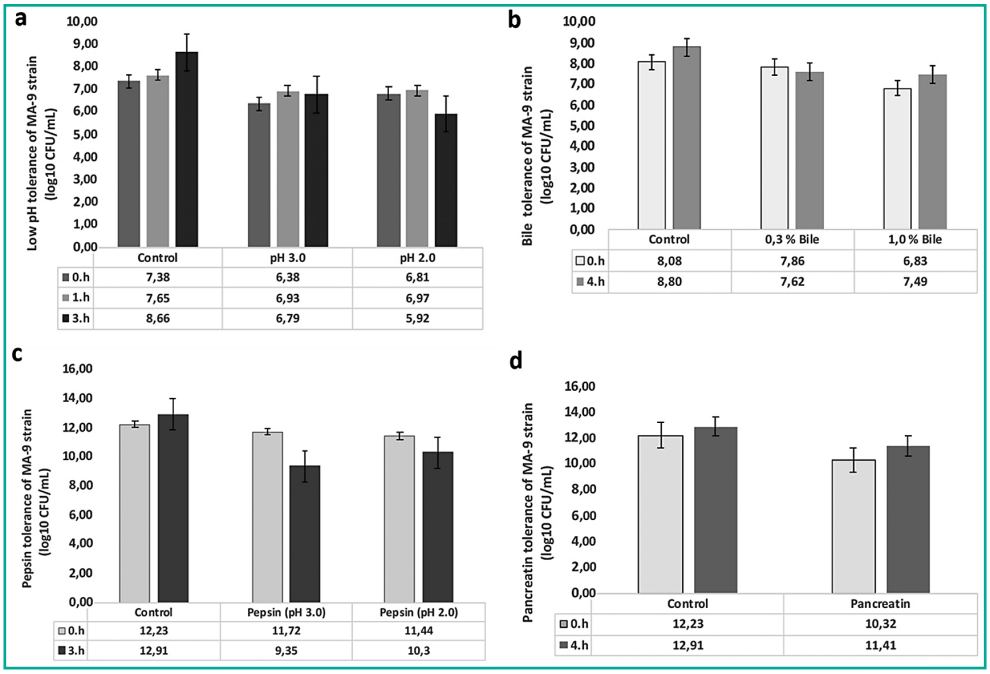

FIGURE 1: *Survival in simulated gastrointestinal conditions of L. delbrueckii MA-9 (log_{10} CFU/mL).* ***a:*** *Resistance to low pH values.* ***b:*** *Resistance to different concentrations of bile.* ***c:*** *Pepsin tolerance of L. delbrueckii MA-9.* ***d:*** *Pancreatin tolerance of L. delbrueckii MA-9.*

pH 3 and 2, but a decrease was observed in the 3rd hour. At the last hour of incubation, there was more live cell loss at the pH 2 than pH 3, but *L. delbrueckii* MA-9 still maintained its cell viability (Figure 1a). The viability of the MA-9 strain decreased by 0.24 CFU/mL after the incubation period in the culture medium containing bile at a concentration of 0.3%, while an interesting increase by 0.66 CFU/mL was observed for 1% bile. The protection and survival of *L. delbrueckii* MA-9 from the toxic effect of bile suggest that this strain has protein expression, possibly related to bile resistance (bile salt hydrolase (BSH)). However, this assessment needs further validation (Figure 1b). Similarly, the strain has been able to preserve its vitality in culture media including pepsin and pancreatin (Figure 1c, d). It was determined that MA-9 strain tolerated different stress conditions of the gastrointestinal tract when all these results were obtained from the assays performed in the simulated medium.

Assessment of auto and co-aggregation

The aggregation ability of the *L. delbrueckii* MA-9 strain was tested spectrophotometrically. The auto-aggregation activity of MA-9 was recorded as 95%. The co-aggregation abilities of various group pathogen test microorganisms varied from 43 to 56% (Table 2). For human origin test bacteria, the highest coaggregation value was determined for E. coli O157:H7 ATCC 43895, while the lowest value was recorded for *S. enteritidis* RSKK 171. For both *S. agalactiae* and *V. alginolyticus* fish pathogen bacteria, co-aggregation value was determined as 49%. The aggregation abilities of LAB are considered an important probiotic feature that supports the host defense mechanism (Abushelaibiet al., 2017; Śliżewska, et al., 2021). Aggregation ability of MA-9 strain indicated that the strain can colonize on host epithelial surfaces and can also prevent pathogenic microorganisms from adhering to these surfaces.

Tolerance to food preservative substances

The susceptibility properties of *L. delbrueckii* MA-9 strain against sodium benzoate and nisin food additives were tested (Figure 2 (a, b, c, and d)). When the spectrophotometric data of the strain for sodium benzoate were examined, it was observed that there was some decrease in vitality as the concentration of sodium benzoate increased (Figure 2a). When the acid production data at the same concentrations were examined, an increase in the pH of the media was determined as the concentration of sodium benzoate increased (Figure 2b). The increase in pH of the media indicates a slight decrease in the strain viability. According to both methods, the strain was determined to maintain its viability even at high concentrations of sodium benzoate. For the 0.1% concentration of sodium benzoate, determined as the general limit of use in foods, live cell counting was performed. According to the obtained data, the viability of the strain decreased by 0.14 log/CFU compared to the control group (Figure 2c). The nisin susceptibility data showed that MA-9 strain growth was only inhibited at the highest concentration of nisin (150 µg/mL) with an inhibition zone of 3.14 ± 0.13 mm (Figure 2d). The strain showed resistance to the other eight concentrations of nisin. The results indicated that the strain was tolerant to the legal limits used in foods and even to the upper concentrations of both additives. Therefore, it can be thought that more viable strains will have an advantage in the digestive system and exceed the gastrointestinal tract barrier.

Screening of strain for enzymatic activities

The enzyme activities of the MA-9 strain were assayed by spot-dropping on agar media. In both media containing starch and lichenan, clear zone formation was observed in the spot-dropping regions. Therefore, the amylase and lichenase enzyme activities of the strain was evaluated as positive. After molecular characterization and purification of amylase and lichenase enzymes by further studies, it may be possible to use MA-9 strain in various fields such as foodstuff, alcoholic beverages, textile industry.

TABLE 2: *Co-aggregation ability of the L. delbrueckii MA-9.*

Auto-aggregation %	Coaggregation %						
	L. monocytogenes ATCC 7644	*E. coli* ATCC 35218	*E. coli* O157: H7 ATCC 43895	*S. enteritidis* ATCC 13076	*S. enteritidis* RSKK 171	*S. agalactiae*	*V. alginolyticus*
95	51	55	56	51	43	49	49

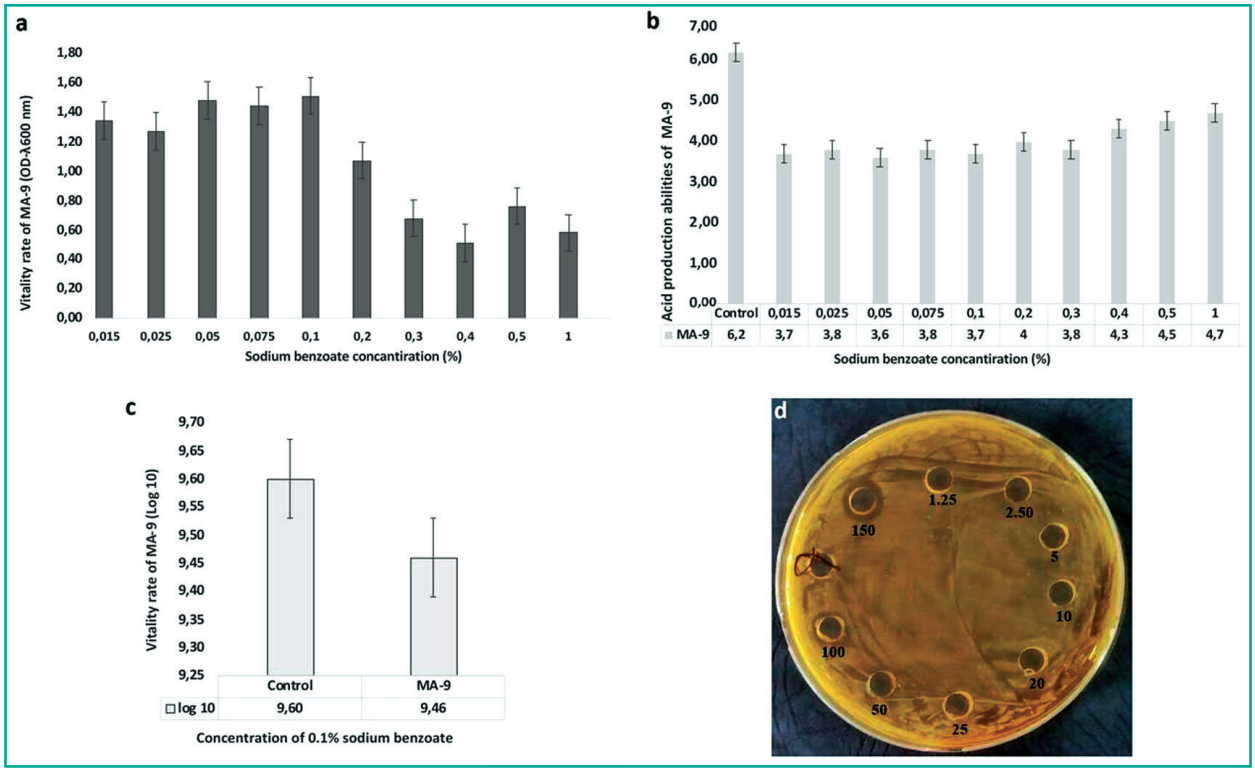

FIGURE 2: *Sodium benzoate and nisin resistance of L. delbrueckii MA-9 strain.* ***a:*** *spectrophotometric data for seven different (0.075–1%) concentrations of sodium benzoate resistance of L. delbrueckii MA-9.* ***b:*** *acid production data of the strain in media containing sodium benzoate.* ***c:*** *Viability of L. delbrueckii MA-9.* ***d:*** *Nisin resistance of L. delbrueckii MA-9 (1.25–150 µg/mL).*

Antimicrobial activity

The inhibitory activity of cell free culture supernatants of MA-9 strain against different group indicator microorganisms was determined using well diffusion method and the results were presented in Table 3. The antibacterial activities of *L. delbrueckii* MA-9 against some human-originated bacteria varied from 2.91 to 10.95 mm. The anti-candidal activity of the strain was determined as 2.73 ± 0.57 mm and 3.22 ± 0.07 mm for *C. albicans* ATCC 10231 and *C. glabrata* RSKK 04019. The inhibitory activity of the strain against various fish pathogens varied from 3.61 to 10.49 mm. The highest inhibitory activity among all different group indicator microorganisms was determined for *S. epidermidis* ATCC 11228 (10.95±0.71), *L. monocytogenes* ATCC 7644 (10.63±0.38) and *A. hydrophila* ATCC 19570 (10.49±1.60), respectively. In our study, no inhibitory activity against only two clinical human-foodborne bacteria (*E. coli* O157:H7 ATCC 43895 and *M. luteus* NRRL B 4375) was recorded among twenty-three test microorganisms. The detailed listing of used twenty-three pathogen test microorganisms has been provided in Table 3.

Zhang et al. (2019) reported a significant increase in growth performance of *Cyprinus carpio* fed with dietary supplementation of *L. delbrueckii.* Also, Zhang et al. (2017) indicated that the same fish species fed with dietary supplementation of *L. delbrueckii* had a 40% reduction in diseases caused by the *A. hydrophila* pathogen bacteria compared to the control group. In our study, *L. delbrueckii* MA-9 strain showed inhibitory activity against all indicator fish pathogens. Therefore, the use of the strain as a feed supplement in aquaculture can be suggested.

TABLE 3: *Antibiotic susceptibility of L. delbrueckii MA-9 strain.*

Test microorganisms Human clinical and food-borne bacteria	*Lactobacillus delbrueckii* MA-9	Inhibition zone diameter[a] (mm)
Bacillus cereus RSKK 863	±	4.01±0.93
Bacillus megaterium (Pasteur Ens.5117)	±	5.07±0.61
Bacillus subtilis RSKK 244	±	3.24±0.47
Enterococcus faecalis ATCC 29212	±	3.08±0.25
Escherichia coli ATCC 11229	±	4.61±0.068
Escherichia coli ATCC 35218	±	3.05±0.07
Escherichia O157:H7 ATCC 43895	–	–[b]
Listeria monocytogenes ATCC 7644	++	10.63±0.38
Micrococcus luteus NRRL B- 4375	–	–
Salmonella enteritidis RSKK 171	±	4.46±0.23
Shigella sonnei MU:57	±	4.06±1.24
Staphylococcus aureus ATCC 25923	±	2.91±0.42
Staphylococcus epidermidis ATCC 11228	++	10.95±0.71
Yersinia enterocolitica ATCC 11175	±	4.83±0.58
Yeast pathogens		
Candida albicans ATCC 10231	±	2.73±0.57
Candida glabrata RSKK 04019	±	3.22±0.07
Bacterial fish pathogens		
Aeromonas hydrophila ATCC 19570	++	10.49±1.60
Lactococcus garvieae	±	6.13±0.37
Streptococcus agalactiae Pas. Inst. 55118	±	4.07±0.08
Vibrio anguillarum A4	±	3.61±0.37
Vibrio anguillarum M1	±	4.66±0.52
Vibrio alginolyticus	±	3.96±0.39
Yersinia ruckeri	±	4.90±0.43

[a]: Diameter of the inhibition zone including disc diameter. Values are reported as means ± SD of three separate experiments. [b]: Indicates no antimicrobial activity. No inhibition (–), inhibition zone diameter value <8 mm (±), inhibition zone diameter value 8–10 mm (+), inhibition zone diameter value > 10 mm (++).

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Antioxidant activity

Lactic acid bacteria contribute to the preservation of colonic epithelial cell integrity and tissue hemostasis by modulating the redox state in the intestine, mucosal surface and mucosa (Sun, et al., 2010). Therefore, in this study, the antioxidant properties of *L. delbrueckii* MA-9 strain were investigated in order to determine its possible therapeutic effect on the host gastrointestinal redox potential. The antioxidant properties of *L. delbrueckii* MA-9 strain were determined using DPPH radical scavenging and ferrous-ion chelating assays. DPPH radical scavenging activity tests the hydrogen donor ability of the strain whose antioxidant properties were investigated. Ferrous-ion chelating activity, on the other hand, is the situation where Fe^{2+} ions, which can be a source of hydroxyl radicals, are partially converted into ferric state (Fe^{3+}) by lactic acid bacteria and rendered unusable in the Fenton reaction region. In this study, it was determined that DPPH free radical scavenging activity of *L. delbrueckii* MA-9 strain was determined as 43.40%, while iron ion chelating activity was not found. It is thought that this result may be due to the differences in the working mechanisms of these methods used. In some recent studies, it has been reported that the DPPH radical scavenging activities of *L. delbrueckii* strains isolated from different sources (fermented yak milk, fermented foods, milk) are in the range of 17.20–35% (Ding, et al., 2017; Cheon et al., 2020; Riane et al., 2021). The per cent DPPH scavenging value we obtained was similar to the literature data, but higher activity was recorded. Although studies on the iron ion chelating activity of various foods or exopolysaccharides obtained by using *L. delbrueckii* strains have been reported in the literature (Jhan et al., 2015; Adebayo-Tayo and Fashogbon, 2020) as far as we know, there is no study on the activity of the strain directly. The data obtained show that the strain has good antiradical activity. Therefore, it is thought that the MA-9 strain may exert therapeutic effects on the host health by modulating the redox state in the gastrointestinal system.

Assimilation of cholesterol

The anti-cholesterol activities of the pellet and supernatant were tested in different bile concentrations and the results were presented in Figure 3. The supernatant of the MA-9 strain had the higher cholesterol assimilation ability at both bile concentrations than the pellet. The highest assimilation value for the supernatants was determined at a concentration of 0.3% bile (82.30%), followed by 1% bile (71.24%) concentration. For the pellets, the anti-cholesterol activity values were 38.05% and 54.65% for 0.3 and 1% bile. Based on the positive *in vitro* results, we may assume that the *L. delbrueckii* MA-9 strain can assimilate dietary cholesterol and stabilize serum cholesterol level by showing a similar activity in the gastrointestinal tract of the host. However, these inferences need to be confirmed by in vivo experiments in future studies.

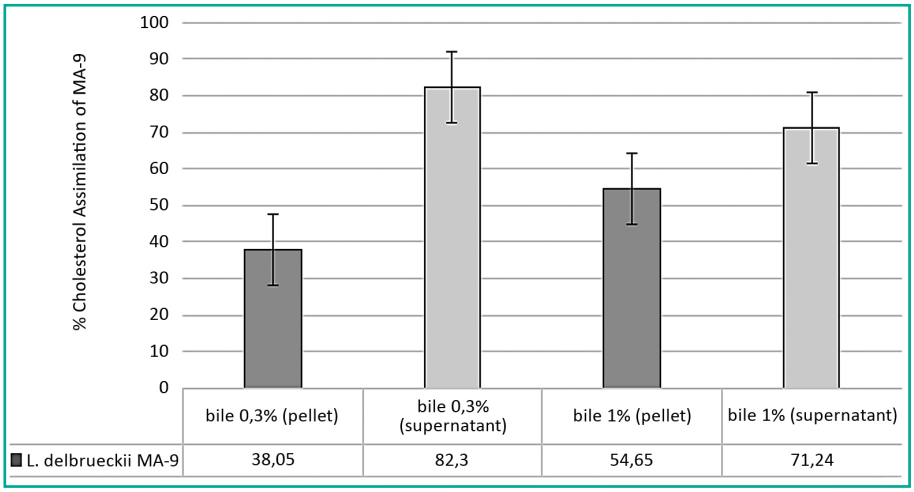

FIGURE 3: *Cholesterol removal ability of L. delbrueckii MA-9.*

Conclusions

In the present study, the non-hemolytic *L. delbrueckii* MA-9 strain was found to be resistant to simulated gastrointestinal system conditions and various food preservatives. This is important in terms of reaching more intestinal cultures and producing various mediators. The strain also showed antimicrobial activity against various clinical human food-borne bacteria, yeast and fish pathogen microorganisms, reduction of the DPHH radical, high levels of cholesterol assimilation activity and aggregation ability. It is thought that the host health can be modulated by using *L. delbrueckii* MA-9 strain as a dietary supplement.

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Conflict of Interest

The authors declare none conflict of interest.

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Address of corresponding author:
Meltem Asan-Ozusaglam
Aksaray University
Faculty of Science and Letters
Department of Molecular Biology and Genetic
68100, Aksaray
Turkey
meltemozusaglam@gmail.com