

A new probiotic candidate bacterium from human milk: *Limosilactobacillus vaginalis* MA-10

M. Asan-Ozusaglam^{1*}  and A. Gunyakti^{1,2}

¹ Department of Biotechnology, Faculty of Science and Letters, Aksaray University, 68100, Aksaray, Turkey

² Institute of Biotechnology, Ankara University, 06110, Ankara, Turkey

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ABSTRACT

The probiotic and technological potentials of lactic acid bacteria originating from human milk are becoming a remarkable research area. In the present study, *Limosilactobacillus vaginalis* MA-10 isolated from human milk was investigated in vitro for its probiotic and technological aspects. According to the results obtained in the study, MA-10 strain exhibited non-haemolytic activity and various degrees of sensitivity to most of the tested antibiotics. The strain showed good resistance to the gastrointestinal system and maintained its viability under these conditions. Its antimicrobial activity against human or clinical bacterial and fungal microorganisms and fish bacteria was determined in the range of 2.38–11.22 mm. The MA-10 strain was able to assimilate cholesterol ranging from 31.42 to 82.30%. The strain showed 9.34% ferrous-ion chelating and 32% DPPH free radical scavenging activities. These initial results from the present study confirm that *L. vaginalis* MA-10 may be a new source with appropriated probiotic and technological traits for various industries, and further in vivo assays. Due to the limited number of studies on *L. vaginalis* strains originated from human breast milk in the literature, the data obtained in this study are thought to be important for revealing the basic probiotic properties of the strain.

KEYWORDS

human milk, antioxidant activity, cholesterol assimilation, gastrointestinal system

* Corresponding author. E-mail: meltemozusaglam@gmail.com

1. INTRODUCTION

A healthy gastrointestinal tract microbiota is highly balanced in terms of the distribution of beneficial/harmful microorganisms, but the deterioration of this balance may lead to the onset of a pathological process called microbial dysbiosis and the development of symptoms related to it. The deterioration of commensal microbiota homeostasis causes a wide range of diseases. Most of the bacterial groups, such as *Lactobacillus*, *Lactococcus*, *Bacillus*, *Bifidobacterium*, which are naturally found in the gastrointestinal tract, are frequently used as food and health-promoting supplements to re-establish homeostasis (Kothari et al., 2019). Also, in recent years, human milk has gained considerable importance as safe and attractive source of probiotic lactic acid bacteria (LAB). The most commonly found and isolated bacteria species from human breast milk include *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, and *Enterococcus*. Human milk bacteria are gaining much attention because of their ability to protect the host from pathogenic microorganisms, survive in the gastrointestinal tract, adhere to epithelial cells of the mucosa, assimilate cholesterol, support immune defence, produce antimicrobial substances and mediators (Amenu, 2014).

The use of LAB in food and feed industrial applications enjoys a “generally recognised as safe” (GRAS) status. Literature have focused on the *Lactobacillus* genus among LAB, and their probiotic effects were reported to be genus-specific, species-specific, or even strain-specific. In the literature, the research is generally focused on the probiotic potential of *Lactobacillus gasseri*, *Lactobacillus fermentum*, and *Lactobacillus plantarum* strains originated from human milk (Gunyakti and Asan-Ozusaglam, 2018, 2019). However, few studies have reported the probiotic potential and technological properties of *Limosilactobacillus vaginalis* isolated from human stomach, feces, vagina, and chicken. Therefore, the aim of the current study was to investigate *L. vaginalis* originated from human milk as a possibly potential probiotic candidate.

2. MATERIALS AND METHODS

2.1. Identification of *Lactobacillus* strains

The Gram-positive and catalase-negative isolate with bacillus morphology was first identified using API 50 CHL (BiomérieuxSA, France) according to the manufacturer’s instructions. The defined strain was then sent to IONTEK (Turkey) for molecular identification by 16S rRNA PCR analysis.

2.2. Determination of safety

Haemolytic activity was verified by inoculating Colombia agar media containing 5% sheep blood (OR-BAK, Turkey) with the isolate and incubating under anaerobic condition at 37 °C for 24 h. Haemolytic activity was determined by zone formation around the colonies. The greenish zone was considered α -haemolysis, clear zone was classified as β -haemolysis, and no zone indicated γ -haemolysis.

The susceptibility profiles of *L. vaginalis* MA-10 were assessed by the disc diffusion method against eleven commercial antibiotics (Oxoid, Germany). The suspension adjusted to Mc Farland 0.5 densities was spread on MRS agar medium. Each antibiotic disc was located on the



inoculated MRS agar medium in triplicate, and the Petri dishes were incubated at 37 °C for 24 h. The inhibition zone diameters were measured and assessed as stated by CLSI (Clinical and Laboratory Standards Institute) guideline (Sharma et al., 2016).

2.3. Determination of probiotic potential and technological properties

2.3.1. Cell viability under simulated gastrointestinal conditions. The resistance of *L. vaginalis* MA-10 to simulated gastric conditions including low pH, bile, gastric, and small intestine juice tolerances were determined as described in our previous study (Gunyakti and Asan-Ozusaglam, 2019).

2.3.2. Determination of antimicrobial activity. Fifteen clinical and food-borne bacteria, two fungal strains and seven fish originated pathogenic bacteria were used to determine the antimicrobial activity of *L. vaginalis* MA-10 using well diffusion method. The indicator microorganisms adjusted to McFarland 0.5 standard were spread onto the specific agar media. MA-10 strain supernatant (100 µL) was placed into each well (7 mm diameter). The plates were incubated for 24 h and then the inhibition zones around the wells were measured using Vernier caliper. The inhibition zone diameter was obtained by subtracting the diameter of the well from the entire inhibition diameter. The experiment was conducted three times.

2.3.3. Cholesterol assimilation activity. *L. vaginalis* MA-10 culture was inoculated into the MRS broth containing 100 µg mL⁻¹ of cholesterol (Sigma-Aldrich, USA) and different concentrations of bile (0.3 and 1%) (Oxoid, UK) then incubated at 37 °C for 24 h. The cholesterol assimilation of supernatant and pellet was separately evaluated using the *o*-phthalaldehyde method (Hosono et al., 1999). Cholesterol removal ability of MA-10 was calculated as described in our previous study (Gunyakti and Asan-Ozusaglam, 2019).

2.3.4. Antioxidant activity. The free radical scavenging activity and ferrous-ion chelating property of MA-10 strain were measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as described in our previous study (Gunyakti and Asan-Ozusaglam, 2019).

2.4. Statistical analysis

Statistical analysis was performed with the Mann-Whitney U-test to identify significant differences in antibiotic susceptibility and antimicrobial activity assay results. The differences were considered significant at a *P*-value of <0.05. The statistical analyses were conducted using SPSS version 22 (SPSS Inc, Chicago, IL, USA).

3. RESULTS AND DISCUSSION

3.1. Identification of *Lactobacillus* strain

The isolated strain was identified by API 50 CHL System as *Lactobacillus acidophilus* (98.7%). Genotypic identification according to the 16S rRNA sequences showed that MA-10 strain was *L. vaginalis*. However, molecular identification with 16S rRNA of strains may be more consistent



than microbial and biochemical techniques. Therefore, the strain was identified as *L. vaginalis* MA-10 according to the 16S rRNA PCR analysis results.

3.2. Determination of safety

The strain had no-zones around the colonies on the blood agar. *L. vaginalis* MA-10 exhibited γ -haemolytic or non-haemolytic activity. Haemolytic activity is related to virulence factors that can be found in pathogenic microorganisms, so the microorganisms under investigation for probiotic properties are expected to be non-haemolytic strains (FAO/WHO, 2002). In this study, the absence of a lysis region for MA-10 strain indicates the safety of this strain as a probable probiotic candidate in food and other application areas.

L. vaginalis MA-10 showed susceptibility to chloramphenicol, amoxicillin, erythromycin, and ampicillin and resistance to amikacin, gentamicin, kanamycin, nalidixic acid, and ofloxacin (Table 1). The intermediate resistance was determined against cloxacillin and penicillin G. There are mean differences in the antibiotic susceptibility of MA-10 strain against the tested antibiotics. The statistical analysis, however, indicated that the inhibition zone averages of MA-10 strain were not statistically significant at the significance level of 0.05 ($P > 0.05$). The safety concerns related to the use of antibiotic-resistant strains as probiotics are due to the possibility of transferring antibiotic-resistance genes to pathogens in the intestine (Curragh and Collins, 1992). However, in the literature, the antibiotic resistance of some LAB is thought to be natural or intrinsic, so chromosomally encoded and thereby non-transmissible. For example, *Bifidobacterium* strains resistant to various antibiotics in quinolone and aminoglycoside groups are used as starter cultures in food applications. Resistance to aminoglycoside antibiotics, such as amikacin, kanamycin, streptomycin, and gentamicin, is considered to be intrinsic in the *Lactobacillus* genus. The reason for the resistance is the lack of electron transport mediated by the cytochrome, as it mediates the drug uptake (Danielsen and Wind, 2003). Literature information supports our study data, suggesting that *L. vaginalis* strain resistance genes are structural and the risk of transfer to other microorganisms is low. However, in later studies, these results can be confirmed by molecular-based methods.

3.3. Determination of probiotic potential and technological properties

3.3.1. Cell viability under simulated gastrointestinal conditions. The high tolerance to acidity at pH 2 and 3 was found for MA-10 with a high survival rate after 3 h (Table 2). The survival rate was found to be over 100% for both bile concentrations during the incubation period (4 h) (Table 2). During fasting and after the meal, the pH ranges from 1.5 to 4.5, and the food digestion is completed in about 3 h (Lebeer et al., 2008). MA-10 strain showed high survival rates at pH 3. Delgado et al. (2015) reported almost similar growth rate for two *L. vaginalis* strains (LV121 and LV51) from the human stomach. Herein, MA-10 strain exhibited cell viability as 7.26 and 7.65 CFU mL⁻¹ at pH 2 and 3, respectively, after 3 h. MA-10 strain showed higher viability at pH 2. The strains that pass the acidic barrier of the stomach are then exposed to bile fluid. Therefore, tolerance to bile is another criterion to be a good probiotic. The strain has maintained its viability under different pH and bile conditions.



Table 1. Antibiotic susceptibility profile of *L. vaginalis* MA-10

	Antibiotics										
	AK	AMC	AM	C	OB	E	CN	K	NA	OFX	P
Inhibition zone diameter (mm)	7.38 ^b ± 0.26	26.11 ± 0.91	26.58 ± 1.04	25.31 ± 0.93	19.86 ± 0.86	26.08 ± 1.36	7.66 ± 0.61	-	-	-	19.68 ± 1.56
CLSI ^a	R	S	S	S	IR	S	R	R	R	R	IR

- Indicates no inhibition zone.

^aThe inhibition zones was evaluated as Susceptible (S) > 20, Intermediate (IR) \cong 15–19, Resistant (R) \leq 14.

^bDiameter of the inhibition zone including disc diameter. AK: Amikacin (10 μ g); AMC: Amoxicillin (30 μ g); AM: Ampicillin (10 μ g);

C: Chloramphenicol (10 μ g); OB: Cloxacillin (5 μ g); E: Erythromycin (15 μ g); CN: Gentamicin (10 μ g); K: Kanamycin (30 μ g); NA: Nalidixic Acid (5 μ g); OFX: Ofloxacin; P: Penicillin G (10 μ g).



Table 2. Acid and bile tolerance of *L. vaginalis* MA-10

Acid tolerance (\log_{10} CFU mL^{-1})								Bile tolerance (\log_{10} CFU mL^{-1})					
pH 2				pH 3				0.3% bile			1% bile		
0 h	1 h	3 h	Survival rate (%)	0 h	1 h	3 h	Survival rate (%)	0 h	4 h	Survival rate (%)	0 h	4 h	Survival rate (%)
7.74	7.49	7.26	93.79	8.60	8.23	7.65	88.95	7.37	8.38	113.70	7.97	8.38	105.14

Table 3. Simulated gastric and pancreatic juice tolerance of *L. vaginalis* MA-10

Gastric juice										
pH 2.0 (\log_{10} CFU mL^{-1})			Survival rate (%)	pH 3.0 (\log_{10} CFU mL^{-1})			Survival rate (%)	Pancreatic juice (\log_{10} CFU mL^{-1})		
0 h	3 h	Survival rate (%)		0 h	3 h	Survival rate (%)		0 h	3 h	Survival rate (%)
10.35	10.54	101.83	9.95	9.57	96.18	10.02	10.09	100.69		

L. vaginalis MA-10 also showed good survival ability under simulated gastric and pancreatic conditions (Table 3). In general, most LAB seem to have natural survival ability in pancreatin, but contrary to pancreatin tolerance, bile tolerance is specific to the strain (Masco et al., 2007). *L. vaginalis* MA-10 showed 100.69% survival rate after 3 h under simulated gastric juice conditions at pH 2 and 3. The results indicate that MA-10 can survive under gastrointestinal conditions and can thus be a probable candidate as probiotic strain.

3.3.2. Antimicrobial activity. Antimicrobial activities of MA-10 tested against 25 indicator strains are presented in Table 4. The inhibition zones of the food-borne and clinical bacterial strains varied from 2.38 to 11.22 mm. The highest two inhibitory activities were found against *Staphylococcus epidermidis* ATCC 11228 and *Listeria monocytogenes* ATCC 7644. The strain inhibited the growth of two *Candida* strains and inhibition zone diameters were determined as 2.70 and 2.87 mm. The growth of all fish originated pathogenic bacteria was also inhibited by MA-10 strain with inhibition zones ranging from 3.53 to 7.35 mm. The highest antibacterial activity was determined against *Aeromonas hydrophila*. The statistical analysis indicated that the differences between zone averages of MA-10 were not statistically significant at the significance level of 0.05 ($P > 0.05$).

Anukam and Reid (2007) reported the antibacterial activity of vaginal *L. vaginalis* against *Escherichia coli* O157:H7 and *Staphylococcus aureus* as over 15 mm of inhibition zone diameter. In another study conducted by Siroli et al. (2017), vaginal *L. vaginalis* BC15, BC16, and BC17 showed antibacterial activities against *L. monocytogenes* ATCC 13932 with a 1 to 3 mm inhibition zone diameter. Herein, MA-10 strain showed higher antibacterial activity than these vaginal *L. vaginalis* strains against *L. monocytogenes* ATCC 7644 with 9.79 mm inhibition zone diameter. Intestinal colonisation or infection of the intestine with pathogenic microorganisms may cause a risk of necrotising enterocolitis that can be decreased with the colonisation with lactobacilli and bifidobacteria of the intestine (Reid et al., 2003). The antimicrobial activities of



Table 4. Antimicrobial activity of *L. vaginalis* MA-10

Test microorganisms	<i>L. vaginalis</i> MA-10 Inhibition zone diameter (mm)
Human food borne and clinical	
Bacterial strains	
<i>E. coli</i> ATCC 11229	3.76 ± 0.08 ^a
<i>E. coli</i> O157:H7	-
<i>B. megaterium</i> (Pasteur Ins. 5117)	3.52 ± 0.03
<i>S. enteritidis</i> RSKK 171	3.21 ± 0.16
<i>B. cereus</i> RSKK 863	3.25 ± 0.66
<i>B. subtilis</i> RSKK 244	-
<i>E. coli</i> ATCC 35218	-
<i>M. luteus</i> NRRL B-4375	-
<i>S. enteritidis</i> ATCC 13076	-
<i>S. sonnei</i> MU:57	2.38 ± 0.27
<i>S. aureus</i> ATCC 25923	-
<i>Y. enterocolitica</i> NCTC 11175	-
<i>E. faecalis</i> ATCC 29212	-
<i>L. monocytogenes</i> ATCC 7644	9.79 ± 1.13
<i>S. epidermidis</i> ATCC 11228	11.22 ± 0.76
Fungal strains	
<i>C. albicans</i> ATCC 10231	2.70 ± 0
<i>C. glabrata</i> RSKK 04019	2.87 ± 0.24
Fish originated bacterial strains	
<i>V. alginolyticus</i>	4.64 ± 0.57
<i>V. anguillarum</i> M1	3.73 ± 0.35
<i>V. anguillarum</i> A4	3.53 ± 0.31
<i>A. hydrophila</i> ATCC 19570	7.35 ± 0.78
<i>L. garviae</i>	4.44 ± 0.45
<i>Y. ruckeri</i>	4.88 ± 0.73
<i>S. agalactiae</i> Pas. Inst. 55118	4.71 ± 0.76

- Indicates no antimicrobial activity.

^aDiameter of the inhibition zone including disc diameter.

probiotic strains are necessary to prevent infection or invasion of pathogenic microorganisms. Various LAB are known to synthesize various inhibitory agents such as bacteriocins, organic acids, but in this study, it was observed that the inhibitory activity was lost at neutral pH. Therefore, the inhibitory activity in the current situation can be ascribed to organic acid-like metabolic products from *L. vaginalis* MA-10. These results indicated that MA-10 strain can be used as a feed supplement in aquaculture in the form of single or co-culture with various probiotic microorganisms. Besides, the strain may be a new alternative bio-source that exhibits inhibitory activity against human pathogens.

3.3.3. Cholesterol assimilation activity. The cholesterol-reducing activity of MA-10 was determined in vitro in the presence of 0.3 and 1% bile, the results obtained were 44.69 and 31.42% for pellets and 82.30 and 81.19% for supernatants, respectively. The higher anti-cholesterol activity at both bile concentrations was obtained for supernatants compared to



pellets. The increased bile concentrations caused a small decrease in cholesterol assimilation activities of the pellet and supernatant. The possible cause of cholesterol assimilation activities of *Lactobacillus* strains is to decrease the solubility of cholesterol and therefore reduce cholesterol intake (Oh et al., 2018). The high serum cholesterol level can be a risk factor for cardiovascular disease; therefore, cholesterol-reducing ability has become an important functional feature for the selection of probiotic strains. There is no literature data on the cholesterol-lowering activity of *L. vaginalis* according to our knowledge. Nowadays, the development of cholesterol-lowering bio-therapeutics has become an important area. *L. vaginalis* MA-10 can assimilate with good cholesterol-lowering percentages and, therefore, can be a new bio-agent to reduce cholesterol level.

3.3.4. Antioxidant activity. The antioxidant activity was evaluated by DPPH free radical scavenging and ferrous-ion chelating activities. The strain showed 32% DPPH free radical scavenging and 9.34% ferrous-ion chelating activities. Many LAB have special systems to prevent the organisms from the reactive oxygen species. The activities of various LAB to reduce reactive oxygen species have been induced by many in vitro experiments. However, there are a limited number of studies on *L. vaginalis* strains. Delgado et al. (2015) tested the total antioxidant activity of *L. vaginalis* LV51 and LV121 strains and found 21% and no activity. Several synthetic antioxidants widely used in food, cosmetic, and medicinal industries are carcinogenic and may cause liver damage (Saduakhasova et al., 2013). Antioxidant activity of the probiotic bacteria plays an important role in the protection of host health from free radicals. Therefore, *L. vaginalis* MA-10 can be an alternative to the synthetic antioxidants as a natural antioxidant source.

4. CONCLUSIONS

In the current study, probiotic potential and some technological properties of *L. vaginalis* MA-10 isolated from human milk were examined in vitro. MA-10 strain exhibited appropriate probiotic effects including non-haemolytic activity and high tolerance to gastrointestinal conditions. The results of the antimicrobial, antioxidant, and anti-cholesterol activity assays also indicated that MA-10 strain can beneficially affect host health with these properties. Considering the results of the current study, *L. vaginalis* MA-10 strain may be a new bio-source for food, feed, and pharmaceutical industries after confirming its benefits and safe use in animal models.

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