





CHARACTERIZATION OF SOME PROBIOTIC PROPERTIES OF *LACTOBACILLUS GASSERI* MA-1 FROM HUMAN MILK

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ABSTRACT

Human milk is a source of various lactic acid bacteria with the potential of probiotics. The aim of this study was to screen in vitro some probiotic properties of *Lactobacillus gasseri* MA-1 originated from human milk. The strain showed γ -hemolytic activity (no hemolytic activity) and susceptibility to Chloramphenicol, Cloxacillin and Penicillin G antibiotics. MA-1 with safety properties also exhibited a good tolerance to pH 2 and 0.3% bile conditions. *L. gasseri* MA-1 showed high ability of auto-aggregation (97%). The co-aggregation activities the strain with five human (*Listeria monocytogenes* ATCC 7644, *Escherichia coli* ATCC 35218, *Salmonella enteritidis* ATCC 13076, *Escherichia coli* O157:H7 and *Salmonella enteritidis* RSKK 171) and two fish (*Streptococcus agalactiae* and *Vibrio alginolyticus*) originated bacteria varied from 45% to 57%. The results indicated that, *L. gasseri* MA-1 strain could be a promising candidate for probiotic products.

Keywords: Human breast milk, Tolerance to gastrointestinal condition, Auto-aggregation, Co-aggregation

1. INTRODUCTION

Probiotics are non-pathogenic living microorganisms and have a beneficial effect on the host health when given in enough amounts [1]. In the last decade, research on probiotics has progressed significantly and considerable progress has been made in the selection and characterization of certain probiotic strains [2].

Probiotics tend to provide protection against various enteric pathogens in addition to host microflora. Probiotics also improve the host's intestinal barrier property by competition with pathogenic microbiota for adhesion to the gut and improving their colonization [3]. Since the probiotic property is specific to each strain, each strain should be investigated for survival and specific colonization ability in the human gastrointestinal (GI) system. FAO / WHO guidelines also recommend the detection of an antibiotic resistance pattern for species to determinate safety of the strain (GRAS) [1].

The most studied probiotics belong to the genera *Lactobacillus* and *Bifidobacterium*, which have health support effects in both humans and animals [4, 5], Enterococcus, Micrococcus and Bifidobacterium [6]. *Lactobacillus gasseri* found naturally in human milk, gastrointestinal and vaginal system [7-9] and defined as generally regarded as safe (GRAS) microorganism [10].

Probiotic strains should be able to maintain a sufficient number of viability of the host's gastrointestinal tract [11]. In addition, they have to be adherent to human epithelial cells and able to decrease pathogen microorganism adhesion to surfaces. Other important characteristic of probiotics are that they must be safe [12]. Therefore, the present study was aimed to evaluate *L. gasseri* MA-1 for some desirable features in probiotic microorganisms including safety aspects, survival in gastrointestinal conditions and auto-aggregation and co-aggregation abilities. Other features for probiotic evaluation of MA-1 strain have been published in our previous studies [9, 13].

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2. MATERIALS AND METHODS

2.1. Safety Assessment

L. gasseri MA-1 strain was tested for its antibiotic susceptibility using disc diffusion method [14]. The suspension adjusted to McFarland 0.5 was spreaded onto MRS agar medium (100 µL). Antibiotic discs (Oxoid) were placed on the surface of inoculated agar with three replicates and then incubated for 24 h at 37°C. The diameters of zone surrounding each of the discs were measured by Vernier caliper. The results were presented according to CLSI (Clinical and Laboratory Standards Institute) 2012 standards. The inhibition zone of the strain was considered as susceptible (S) >20, intermediate (IR) \cong 15–19 and resistant (R) \leq 14.

Hemolytic activity of MA-1 strain was assayed on Colombia agar supplemented with sheep blood (0.5%) (OR-BAK, Ankara, Turkey) using the streak-plate method. The plate was then incubated at 37°C for 24 h under anaerobic condition and then evaluated for the hemolytic reaction. *Enterococcus faecalis* ATCC 29212, *E. coli* ATCC 35218 and *Staphylococcus aureus* ATCC 25923 were tested as control.

2.2. Survival in Gastro Intestinal Conditions

2.2.1. Acid tolerance

The tolerance of the strain to the low pH environments was evaluated in vitro against pH 2 and 3 in MRS broth at 37°C. The measurements of spectrophotometric (Beckman Coulter DU 730) were made at 600 nm for 0, 1 and 3 h of incubation. 0.1 mL inoculum from each end of the incubation time was then spread onto MRS agar. The inoculated plates were then incubated under anaerobic conditions at 37°C for 24 h. Viable cells were counted and calculated in Log₁₀ (CFU/mL) [15].

2.2.2. Bile tolerance

The MRS broth containing bile (0.3% and 1%, Oxoid) was inoculated with the strain and then incubated for 4 h at 37°C. The measurements of spectrophotometric were made at 600 nm at 0 and 4 h of incubation twice for each sample. MRS agar media was also inoculated from the test groups after these incubation times and then incubated under anaerobic conditions for 24 h at 37°C. The survival cell was counted after 0 and 4 h and calculated as described acid tolerance assay [16].

2.2.3. Simulated gastric and pancreatic juice tolerance

The tolerance of *L. gasseri* MA-1 to simulate gastric transit was assayed using the simulated gastric solutions containing pepsin (3 g/L, Sigma-Aldrich) at pH 2 and 3. The gastric solution was inoculated with bacterial suspension adjusted to McFarland 0.5 standard (1%) and incubated for 3 h at 37°C. The cell suspensions were inoculated on MRS agar media at different incubation time intervals (0 and 3 h) and then incubated under anaerobic condition for 24 h. The viable cell was counted and expressed as Log₁₀ (CFU/mL) [17].

The simulated small intestine solution was prepared by using pancreatin (1 g/L, Sigma-Aldrich) and bile salt (0.03 g/L, Oxoid) to determine the pancreatic tolerance. The pancreatin solution was inoculated with the culture (McFarland 0.5 standard turbidity, 1%) and incubated at 37°C for 3 h. At 0 and 3 hours of incubated test groups were inoculated onto MRS agar plates and incubated under anaerobic condition for 24 h at 37°C. After the incubation, the viable cell count was calculated in the same manner as before described in the tolerance to gastric juice.

2.3. Auto-aggregation and Co-aggregation Activities

Auto-aggregation and co-aggregation activities of the strain were tested according to Xu et al. [18] with some modifications. The cell suspension (0.6 ± 0.02 at OD₆₀₀ nm) prepared in PBS buffer was incubated at 37°C for 4 h without any moving using a spectrophotometer at OD 600 nm. The percentage of auto-aggregation was calculated as follows:

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

OD1: Pre-incubation optical density, OD2: Optical density after incubation

The co-aggregation ability of MA-1 strain with various pathogen microorganisms such as *L. monocytogenes* ATCC 7644, *E. coli* ATCC 35218, *E. coli* O157:H7, *S. enteritidis* ATCC 13076, *S. enteritidis* RSKK 171, *S. agalactiae* and *V. alginolyticus* was assayed. Equal volumes (2 mL) aliquots of *L. gasseri* MA-1 culture and pathogenic microorganisms (0.6 ± 0.02 at OD₆₀₀ nm) were mixed and then incubated at 37°C for 4 h. After incubation, the sample (0.1 mL) was suspended in PBS buffer (3.9 mL) and read at OD₆₀₀ nm.

The percentage of co-aggregation was calculated as follows:

$$\text{Co - aggregation \%} = \frac{(\text{OD1} + \text{OD2}) - 2(\text{OD3})}{(\text{OD1} + \text{OD2})} \times 100$$

OD1: MA-1 strain optical density (pre-incubation), OD2: Pathogen strain optical density of (pre-incubation), OD3: Mixed strains optical density (after 4 h incubation).

3. RESULT AND DISCUSSION

3.1. Safety Properties of MA-1 Strain

Hemolytic activity and antibiotic resistance profiles of a probiotic candidate are the principal criterion to select safe probiotic strains. One of the most important safety traits for a probiotic strain is the absence of hemolytic activity. In vitro evaluation of hemolytic activity on blood agar media is highly recommended, even for GRAS status bacterial species [12]. *L. gasseri* MA-1 strain showed γ -hemolytic activity (no hemolytic activity) (Figure 1).

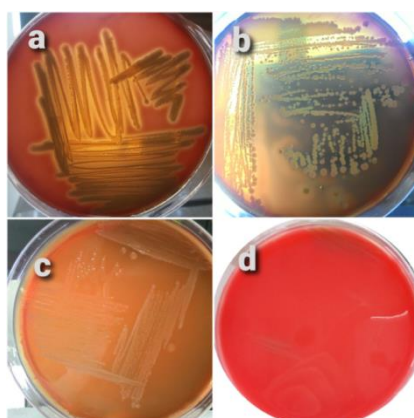


Figure 1. Hemolytic activity of *L. gasseri* MA-1

- a: *E. coli* ATCC 35218 (α -hemolytic)
- b: *S. aureus* ATCC 25923 (β -hemolytic)
- c: *E. faecalis* ATCC 29212 (γ -hemolytic)
- d: *L. gasseri* MA-1 (γ -hemolytic)

Another important requirement to select safe probiotic strain is the lack of antibiotic resistance [19]. Antibiotic susceptibility of MA-1 strain is presented in Table 1. The strain was susceptible to Chloramphenicol, Cloxacillin and Penicillin G and resistant to Amikacin, Nalidixic Acid and Ofloxacin (Table1). Although the species *L. gasseri* is evaluated as the GRAS status, antibiotic resistance of the probiotic candidate strain must be tested at the strain level. It is required that probiotic candidate strains do not carry any transferrable antibiotic resistance gene that may be transferred to pathogenic microorganisms [20]. Conversely, intrinsic antibiotic resistance can be evaluated beneficial to the host, to keep useful microbiota living in the gastrointestinal system during a treatment of antibiotic [21]. In the reports, the antibiotic resistance of *Lactobacillus* strains is recognized to be intrinsic or natural because of chromosomally encoded and considering as non-transferable [22]. It is regarded that resistance to aminoglycoside antibiotics, for instance Amikacin, Streptomycin, Kanamycin and Gentamicin is to be intrinsic for the *Lactobacillus* genus. The resistance occurs due to the lack of cytochrome mediated electron transport mediating drug uptake [22].

Table 1. Antibiotic resistance of *L. gasseri* MA-1 strain

Antibiotic discs	<i>Lactobacillus gasseri</i> MA-1	
	CLSI ^a	Mean ± standard deviation
AK (10 µg)	R	- ^b
C (10 µg)	S	20.72 ^c ±0.91
OB (5 µg)	S	23.78±0.85
NA (5 µg)	R	-
OFX (5 µg)	R	-
P (10 µg)	S	31.69±2.41

^a: CLSI, Clinical and Laboratory Standards Institute. ^b: no inhibition zone ^c: inhibition zone diameter. **AK**: Amikacin, **C**: Chloramphenicol, **OB**: Cloxacillin, **NA**: Nalidixic Acid, **OFX**: Ofloxacin, **P**: Penicillin G, **R**: Resistant, **S**: Sensitive

3.2. Tolerance to Gastro Intestinal Conditions

The survival ability of the strain under highly acidic environments and tolerance to bile salts during transition through the gastrointestinal system are two important key factors to be a probiotic candidate [23, 24]. The survival rate of MA-1 strain in acidic conditions showed variability (Table 2). The survival rate of the strain at pH 2 was found as 148.02% after the incubation period (3 h). However, the strain presented lower survival rate at pH 3 (94.60%) than pH 2 condition. The strain has never lost its viability at different pH conditions. Oh et al. [25] reported the survival rate of five *L. gasseri* strains from infant feces as varying from 97.2 to 100.9% at pH 3 after 2 h. In the present study, MA-1 strain showed lower viability at pH 3 (94.60 %) but higher survival rate (148.02 %) at pH 2 after 3 h.

Probiotic strains are exposed to bile fluid after passing the stomach acidic barrier. The survival rate of MA-1 strain was determined as 112.55% at 0.3% bile after 4 h incubation. However, the survival rate slightly reduced (98.29%) with increased concentration (1%) of bile salts (Table 2). Bile plays a primary role in the specific and nonspecific defense system of the gut and therefore, the bile tolerance is evaluated as an important characteristic of probiotic strains [26]. Bile salt tolerance may be due to the ability of bacteria to deconjugate bile salts, which is dependent on the ability of the bacteria to assimilate cholesterol from intestinal medium [27]. In our previous study, *L. gasseri* MA-1 strain also showed high anticholesterol activity (83.41%) at 0.3% bile concentration [13].

Table 2. Acid and bile resistance of *L. gasseri* MA-1 strain

Strain	Acid Tolerance (log ₁₀ CfU/mL)								Bile Tolerance (log ₁₀ CfU/mL)					
	pH 2			Survival rate (%)	pH 3			Survival rate (%)	0.3% bile			1% bile		
	0.h	1.h	3.h		0.h	1.h	3.h		0.h	4.h	Survival rate (%)	0.h	4.h	Survival rate (%)
<i>L.gasseri</i> MA-1	6.8 3	8.72	10.1 1	148.02	6.30	6.33	5.96	94.60	5.1 8	5.83	112.55	4.6 8	4.6 0	98.29

Tolerance to pepsin and pancreatin is evaluated as other principal aspects to the determine survival of the strain in the gastrointestinal conditions [28]. *L. gasseri* MA-1 strain also exhibited good survival ability to the simulated gastric and pancreatic conditions (Table 3). The MA-1 strain showed 83.06 % and 90.53% survival rate at the simulated gastric juice conditions at pH 2 and 3 after 3 h incubation. *L. gasseri* MA-1 showed 89.27% survival rate at pancreatic juice condition after 3 h. The results indicated that *L. gasseri* MA-1 strain could continue viability under gastrointestinal conditions. All the results confirmed that MA-1 strain can be a candidate to be a good probiotic strain.

Table 3. Simulated gastric and pancreatic juice resistance of *L. gasseri* MA-1

Strain	Gastric Juice						Pancreatic juice (log ₁₀ CfU/mL)		Survival rate (%)
	pH 2.0 (log ₁₀ CfU/mL)		Survival rate (%)	pH 3.0 (log ₁₀ CfU/mL)		Survival rate (%)	0.h	3.h	
	0.h	3.h		0.h	3.h				
<i>L.gasseri</i> MA-1	9.86	8.19	83.06	8.66	7.84	90.53	8.67	7.74	89.27

3.3. Auto-aggregation and Co-aggregation Activities

The auto-aggregation and co-aggregation activities of a probiotic candidate strain are primary since auto-aggregation is a relation with adhesion to epithelial cells [29], while co-aggregation states a defensive barrier against pathogenic microorganism colonization [30, 31]. The defensive barrier does not allow pathogen colonization in the human gut [32]. *L. gasseri* MA-1 showed good auto-aggregation ability (97%) (Table 4). The coaggregation activity of MA-1 strain with five human (*L. monocytogenes* ATCC 7644, *E. coli* ATCC 35218, *E. coli* O157:H7, *S. enteritidis* ATCC 13076 and *S. enteritidis* RSKK 171) and two fish (*S. agalactiae* and *V. alginolyticus*) originated bacteria ranged from 45% to 57% (Table 4). Pino et al. [33] indicated the rate of auto-aggregation and co-aggregation with *E. coli* 555 of three vaginal *L. gasseri* strains (F5, W14 and W18) as 6.21-12.23% and 6.35-14.18%. *L. gasseri* MA-1 with high aggregation abilities may be a good barrier to pathogens microorganisms.

Table 4. Auto-aggregation and co-aggregation activities of *L. gasseri* MA-1

<i>L. gasseri</i> MA-1	Autoaggregation (%)	Coaggregation (%)						
		<i>L. monocytogenes</i> ATCC 7644	<i>E. coli</i> ATCC 35218	<i>E. coli</i> O157:H7	<i>S. enteritidis</i> ATCC 13076	<i>S. enteritidis</i> RSKK 171	<i>S. agalactiae</i>	<i>V. alginolyticus</i>
	97	53	56	57	53	45	51	51

4. CONCLUSION

L.gasseri MA-1 isolated from human milk was studied to evaluate its potential probiotic properties. MA-1 strain exhibited good resistance to gastrointestinal system conditions with high survival rate. The strain with high auto-aggregation and co-aggregation activities can be a defensive barrier against pathogenic microorganism colonization. *L.gasseri* MA-1 also showed safety aspects. Therefore, *L.gasseri* MA-1 can be evaluated as a potential bioactive ingredient for food and pharmaceutical industries.

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