



Investigation of *Lactobacillus gasseri* ma-2 as probiotic candidate

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Abstract

Characterization of lactic acid bacteria in human milk has become an interesting field for developing new probiotics. Therefore, some probiotic properties of *Lactobacillus gasseri* MA-2 strain isolated before from human breast milk were screened in vitro. MA-2 strain exhibited safety aspects with no hemolytic activity (γ -hemolytic activity) and sensitivity to penicillin G, cloxacillin and chloramphenicol. The remarkable tolerance to various acid, bile, pepsin and pancreatin conditions was determined with high survival rate. The strain also presented significant auto-aggregation (93%) as well as co-aggregation activity with pathogenic microorganisms. *L. gasseri* MA-2 originated from human milk exhibited promising probiotic properties.

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1. Introduction

Human breast milk is known as the primary source for healthy and rapidly growth of newborns. It includes high amounts of necessary nutrients, prebiotic substances and mutualistic, commensal and potentially probiotic bacteria for infants [1,2]. The microorganism content in human milk is affected by microorganisms in the mother's gut transported by dendritic cells and macrophages from the lymphatic system through the mammary glands and on the surface of the breast [3, 4]. Human milk generally contains 10^3 – 10^5 CFU/ml viable bacteria [5]. Commonly isolated bacteria genera from human milk are including *Bifidobacterium*, *Staphylococcus*, *Enterococcus*, *Streptococcus* as well as *Lactobacillus* [3]. Some strains in milk belonging to lactic acid bacteria (LAB) like *Lactobacillus gasseri*, *L. fermentum*, *L. salivarius*, *Bifidobacterium longum* subsp. *infantis*, *B. breve* have been indicated as promoting normal gut bacterial colonization and preventing diarrhea [6-8]. LAB originated from human breast milk have been interesting because of their probiotic potential, ability to remain in the intestine as well as being human origin [9]. Human milk is a natural source of a potential probiotic or biotherapeutic LAB. *L. gasseri* strains in human milk are considered as the potential probiotic bacteria and used in various commercial probiotic products [9].

Probiotics are living microorganisms when used in sufficient amounts and provide health benefits to the host [10]. Being safe is the most important criteria to evaluate a microorganism as a probiotic culture [11]. The other selection criteria are survival in stomach acidity and bile salts [12]. They should also be resistant to pepsin and pancreatic juice [13]. Adherence to mucus and/or human epithelial cells and activity to reduce pathogen adhesion to surface are the main used in vitro assays for probiotic candidate microorganism [14]. The aim of this study was to evaluate the probiotic potential of *L. gasseri* MA-2 strain previously isolated from human breast milk and characterized some biotechnological aspects [15].

2. Materials and Methods

2.1. Safety evaluation

The hemolytic property was tested on Colombia agar plate containing 5% (w/v) sheep blood (OR-BAK). The inoculated plate was incubated for 24 h at 37°C and then examined for the hemolytic activity. The formation of clean and greenish zones on the plates was evaluated as β -hemolysis and α -hemolysis. No zones around the colonies were determined as γ -hemolytic (non-hemolytic activity).

The antibiotic susceptibility was determined using disc diffusion method. The culture adjusted to Mc Farland

0.5 was streaked on MRS-Agar plates. Antibiotic discs (Oxoid) were put on the inoculated plates and then incubated at 37°C for 24 h. The antibiotic susceptibility aspect of the strain was tested using amikacin (AK, 10 µg/disc), chloramphenicol (C, 10 µg/disc), cloxacillin (OB, 5 µg/disc), nalidixic acid (NA, 5 µg/disc), ofloxacin (OFX, 5 µg/disc), penicillin G (P, 10 µg/disc). The diameter of the inhibition zone was measured using Vernier caliper. The results were examined according to Clinical and Laboratory Standards Institute (CLSI, 2012) standards as susceptible (S) >20 mm, intermediate (IR) ≈ 15–19 mm, resistant (R) ≤ 14 mm [16].

2.2. Determination survival in gastrointestinal conditions

2.2.1. Tolerance to low pH conditions

The tolerance of MA-2 strain to low pH environments was screened in vitro at different values in MRS broth. The overnight culture (16-18 h incubation) of the strain (1%) was inoculated in MRS broth (pH 2 and 3) and incubated at 37°C. 0.1 ml inoculum was taken from each tube at the end of 0, 1 and 3 h incubation time. The inoculums were then diluted and plated on MRS agar. The plates were incubated under anaerobic conditions at 37°C for 24 h for the determination of cell viability. The viable cells were counted and presented as Log₁₀ values of colony forming units per milliliter (CFU/ml) [17].

2.2.2. Tolerance to bile salt conditions

The bile tolerance of the strain was determined in MRS broth containing 0.3% and 1% of bile (Oxoid). The overnight culture (1%) was inoculated in MRS broth containing bile and then incubated for 4 h at 37°C. The cultures were withdrawn at 0 and 4 h interval and diluted. The cell suspension was plated on MRS agar. The plates were incubated under anaerobic conditions for 24 h at 37°C. The tolerance to bile was determined by enumerating the viable cells on the agar plate and calculated in Log₁₀ (CFU/ml) [18].

2.2.3. Tolerance to simulated gastric and pancreatic juice

The tolerance of MA-2 strain to simulate gastric transit was performed using the simulated gastric solution containing 3 mg/ml pepsin (Sigma-Aldrich) adjusted to pH 2 and 3. The gastric solution was inoculated (1%) with the bacterial suspension (McFarland 0.5 standard) and incubated at 37°C for 3 h. The viable cell count was determined on MRS agar plates by the spread of before and after incubation of the suspension samples after 24 h incubation under anaerobic condition. The

viable cell was counted and the percentage survival of the bacteria was calculated in Log₁₀ (CFU/ml) [19]. The survival rate was calculated using the following equation:

$$\text{Survival rate (\%)} = \frac{\text{After incubation Log CFU/ml}}{\text{Before incubation Log CFU/ml}} \times 100 \quad (1)$$

For pancreatic tolerance, the simulated intestine fluid prepared by dissolving pancreatin (1 mg/ml, Sigma-Aldrich) and bile salt (0.03 mg/ml, Oxoid) was inoculated with MA-2 culture (McFarland 0.5 standard turbidity, 1%) and incubated at 37°C for 3 h [19]. The viable cell count and survival rate were determined in the same manner as for the determination of the gastric juice tolerance.

2.3. Abilities of auto-aggregation and co-aggregation

The ability of MA-2 strain to auto aggregate and co-aggregate were obtained according to Xu et al. [20]. The overnight culture of the strain was harvested by centrifugation and washed with PBS twice. Then, the cell suspension prepared in PBS buffer was adjusted to 0.6 ± 0.02 at OD₆₀₀ nm. The sample was incubated without any movement at 37°C for 4 h. After the incubation, the sample (0.1 ml) was then suspended in PBS buffer (3.9 ml). The suspension was read at 600 nm using a spectrophotometer (Beckman Coulter). The auto-aggregation was calculated using the formula:

$$\text{Auto - aggregation \%} = \frac{\text{OD1} - \text{OD2}}{\text{OD1}} \times 100 \quad (2)$$

where OD1 represents pre-incubation absorbance and OD2 represents after incubation absorbance

The co-aggregation ability of the strain was assayed using 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*. The equal volumes (2 ml) of *L. gasseri* MA-2 prepared as described above for auto-aggregation test and each pathogenic bacterium were mixed and incubated at 37°C for 4 h. After incubation period, 0.1 ml of mixed suspension, resuspended in 3.9 ml of PBS buffer and read at OD₆₀₀ nm in the spectrophotometer. The co-aggregation ability was calculated with the following formula:

$$\text{Co - aggregation \%} = \frac{(\text{OD1} + \text{OD2}) - 2(\text{OD3})}{(\text{OD1} + \text{OD2})} \times 100 \quad (3)$$

where OD1 presents pre-incubation absorbance, OD2 presents pre-incubation absorbance of pathogen strain

and OD3 presents absorbance of mixed strains (after 4 h).

3. Results and Discussion

3.1. Safety aspects of *L. gasseri* MA-2 strain

The strain showed no clear or greenish zones on the blood agar around their colonies (Figure 1). MA-2 strain was therefore evaluated as γ -hemolytic or non-hemolytic. Absence of hemolytic activity is evaluated as safe precondition for the selection of probiotic strain [12].

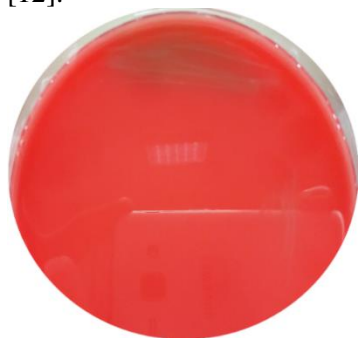


Fig. 1. γ -hemolytic activity of *L. gasseri* MA-2.

Table 1. Antibiotic susceptibility of *L. gasseri* MA-2

<i>L. gasseri</i> MA-2	Inhibition zone diameter (mm)					
	AK	C	OB	NA	OFX	P
Mean \pm standard deviation	- ^a	20.72 \pm 0.91 ^b	23.68 \pm 2.40	-	-	33.19 \pm 1.26
CLSI ^c	R	S	S	R	R	S

^a: no inhibition zone. ^b: the inhibition zone diameter including disc diameter ^c: Clinical and Laboratory Standards Institute

AK: Amikacin, C: Chloramphenicol, OB: Cloxacillin, NA: Nalidixic Acid, OFX: Ofloxacin, P: Penicillin G, R: Resistant, S: Sensitive

3.2. Tolerance to Gastrointestinal Conditions

The viability in the gastrointestinal system should be approved by in-vitro studies prior to use a bacterium as probiotic candidate [24]. Therefore, the strain should be able to resist low pH values of gastric juice and resistant to bile salts. The pH of the gastric juice is considered to be one of the main criteria affecting the viability of probiotic bacteria in the transition from the stomach to the intestine [25]. In the study, the tolerance of MA-2 strain to different pH conditions (pH 2 and 3) was determined. From the data, the strain showed remarkable survival with 4.64 CFU/ml and 5.52 CFU/ml when exposure to pH 2 and pH 3 after the incubation period (3 h) (Figure 2). The strain has never lost its survival at both tested pH conditions. The survival rate of MA-2 strain was determined as 74% and 87.75% after exposure to pH 2 and pH 3. Singroha et al. [26] reported the survival of nine *L. gasseri*

The antibiotic susceptibility assay results indicated that the strain was sensitive to chloramphenicol and cloxacillin, in addition to penicillin G (Table 1). In the reports, *Lactobacillus* is generally susceptible to ampicillin and penicillin (beta-lactam antibiotics) and broad-spectrum antibiotics [21]. *L. gasseri* MA-2 had resistance to amikacin, nalidixic acid and ofloxacin. Probiotic bacteria are capable of harboring intrinsic and mobile genetic elements that provide resistance to various antibiotics. Probiotics added to diets can form an antibiotic-resistant gene reservoir in the human intestine and transfer to pathogens found in the same habitat [22]. In the literature, the antibiotic resistance of *Lactobacillus* strains is recognized to be intrinsic or natural encoded on the chromosome and recognized as non-transferable. The resistance of *Lactobacillus* genus to aminoglycoside antibiotics, such as Amikacin, Streptomycin, Kanamycin and Gentamicin is regarded as intrinsic because of their lack of cytochrome-mediated electron transport mediating drug uptake [23].

strains obtained from ATCC at pH 2 after 3 h as varying from 8.7 CFU/ml to 9.1 CFU/ml. However, Oh et al. [27] indicated that *L. gasseri* 4M13 strain from infant feces showed 6.44 CFU/ml of viability at pH 3 after 2 h.

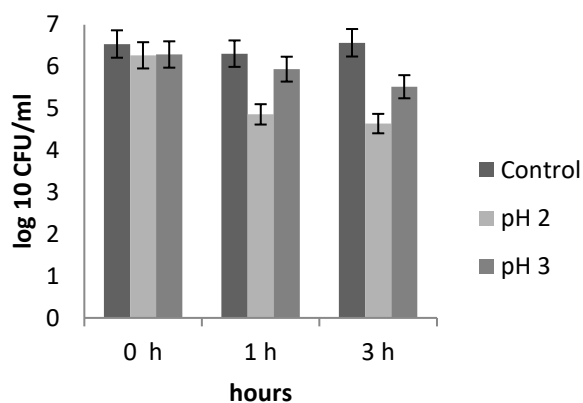


Figure 2. Acid tolerance of *L. gasseri* MA-2.

Bile plays an essential role in the intestinal tract related with non-specific and specific defense mechanisms, and the significance of the inhibitory effect is fundamentally determined by the concentration of bile salts. The bile tolerance is therefore considered an important feature of probiotic candidate LAB strains which allows them to survive and continue their activities in the gastrointestinal system [28]. MA-2 strain was exposed to different bile conditions (0.3% and 0.1%) at the incubation period of 4 h. The strain showed good viability with 7.02 CFU/ml and 5.36 CFU/ml at 0.3% and 0.1% bile concentrations after 4 h incubation (Figure 3). The viability of *L. gasseri* 4M13 from infant feces was reported as 6.45 CFU/ml after 6 h at 1% bile condition [27].

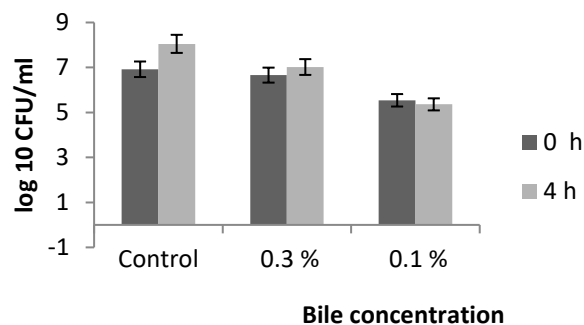


Figure 3. Bile tolerance of *L. gasseri* MA-2.

MA-2 strain was also assayed for colonization in the gastrointestinal system by investigating its viability in the simulated gastric and pancreatic conditions. The strain exhibited survival in both gastric and pancreatic conditions which indicates colonizing the intestines. The viable cell count of the strain showed that there was a bit decrease in the survival at pH 2 gastric juice conditions with 93.33% survival rate (Table 2). However, at pH 3 condition, the strain exhibited higher survival rate (103.62%) than pH 2 gastric environments. MA-2 strain also showed remarkable survival rate (95.43%) at pancreatic juice condition after 3 h. The data indicated that *L. gasseri* MA-2 strain exhibited good survival ability under gastrointestinal conditions. Therefore, MA-2 strain could be potential candidate to evaluate a probiotic strain.

Table 2. Simulated gastric and pancreatic juice tolerance of *L. gasseri* MA-2

Gastric Juice				Pancreatic juice			
pH 2.0 (log ₁₀ CFU/ml)		Survival rate (%)	pH 3.0 (log ₁₀ CFU/ml)		Survival rate (%)	Survival rate (%)	
0.h	3.h		0.h	3.h		0.h	3.h
8.69	8.11	93.33	7.72	8.00	103.62	8.97	8.56

3.3. Auto-aggregation and co-aggregation abilities of *L. gasseri* MA-2 strain

Auto-aggregation and co-aggregation activities of a probiotic strain are important probiotic properties. MA-2 strain exhibited a strong auto-aggregating ability (93%) (Figure 4). The co-aggregation of the strain with five human (*E. coli* ATCC 35218, *L. monocytogenes* ATCC 7644, *E. coli* O157:H7, *S. enteritidis* RSKK 171 and *S. enteritidis* ATCC 13076) and two fish originated bacteria (*V. alginolyticus* and *S. agalactiae*) was also examined. The co-aggregation ability of the strain varied from 36% to 51% (Figure 4). *L. gasseri* MA-2 demonstrated marked co-aggregation (51%) with *S. enteritidis* ATCC 13076 among the

tested bacteria. Auto-aggregation of probiotic strains is related with adhesion to intestinal epithelial cells to survive in the gastrointestinal system [29]. The co-aggregation ability of probiotic strains may allow the formation of a defensive barrier by preventing colonization of pathogenic bacteria [30]. Auto-aggregation properties together with co-aggregation abilities with pathogens are the important criterions in the formation of biofilm protects the host from pathogen colonization [31]. *L. gasseri* MA-2 with high aggregation capabilities can be a good barrier to pathogen microorganisms.

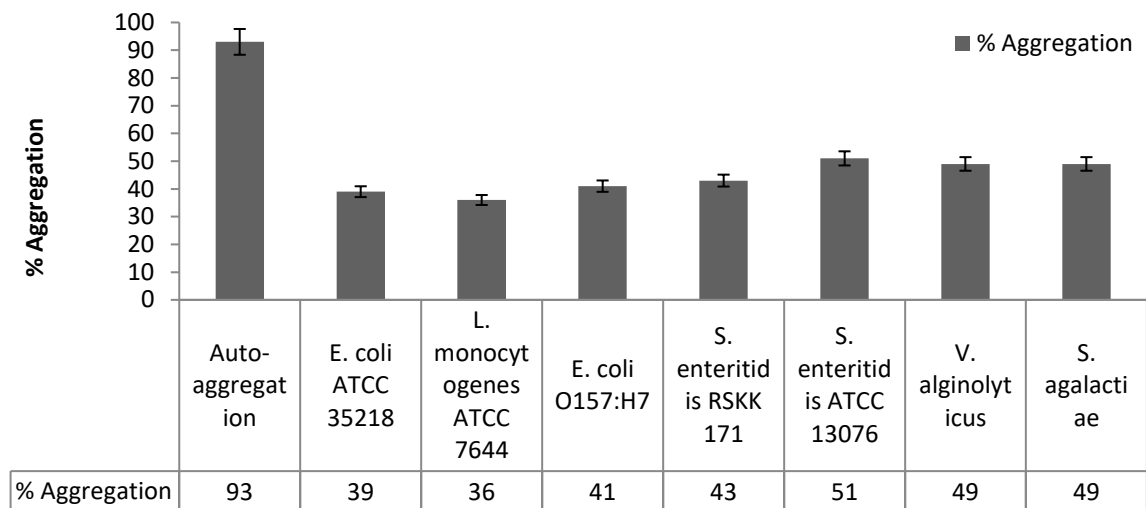


Figure 4. Auto-aggregation and co-aggregation activities of *L. gasseri* MA-2.

4. Conclusions

L. gasseri MA-2 originated from human milk was investigated for its potential probiotic aspects. MA-2 strain could survive in the simulated gastric environment such as acid, bile, pepsin and pancreatin. The strain showed a good ability to grow and survive well in the gastrointestinal conditions with high survival rate. The strain could form defense system for host with high auto-aggregation and co-aggregation abilities. The results showed that *L. gasseri* MA-2 strain with safety features is a suitable candidate for food and pharmaceutical industries as bioactive supplement to promote health and continue the natural balance in the gastrointestinal system of host.

Conflicts of interest

The authors state that did not have conflict of interests.

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