

Assessment of Potential Probiotic Bacteria Isolated from Breast Milk

¹Nayra S.H. Mehanna, ¹Nabil F. Tawfik, ^{1,2}Moussa M.E. Salem,
¹Baheer A.M. Effat and ¹D.A. Gad El-Rab

¹Department of Dairy Science, National Research Center, Dokki, Giza, Egypt

²Department of Biology, Faculty of Sciences and Arts, Khulais,
King Abdulaziz University, Jeddah, Saudi Arabia

Abstract: Breast milk is an important factor in the initiation, development and composition of the neonatal gut microbiota. Human breast milk contains potentially probiotic lactic acid bacteria. For these reasons, the authors isolated lactic acid bacteria from milk of healthy mothers. A total of two hundred and fifty strains of lactic acid bacteria from the genera *Lactococcus*, *Lactobacillus* and *Enterococcus*, isolated from Egyptian Human milk from different areas in Sinai were identified at genus level using physiological characteristics. Eighteen isolates with functional characteristics typical to probiotic bacteria were identified at species and subspecies level by both classical phenotypic and API 50 CHL kits and API 20 STRP. 5 of them belonged to lactic acid cocci and others were referred to genus *Lactobacillus*. Among lactic acid cocci *Enterococcus faecium* (3 strains) were dominant followed by *Lactococcus lactis* (2 strains). Concerning genus *Lactobacillus*, *L. plantarum* and *L. rhamonusus* (4 strains for each) was the most common species followed by *L. casei* and *L. fermentum* (2 strains for each) while *L. acidophilus* comprising (1 strain) of lactobacilli isolates. All these strains were found to resistant to both low pH and 2% bile salt. Moreover, all cultures exhibited a broad spectrum of antagonistic activity against spoilage and pathogenic bacteria. The prebiotic potential of green bell pepper, artichoke leaves and green tea extracts were assessed. Results showed that all of them had a stimulating effect on growth and viability of all tested probiotic bacteria.

Key words: Breast milk • Lactic acid bacteria • Probiotics • Prebiotics

INTRODUCTION

During some months, breast milk is the best food for the rapidly-growing infant since breast feeding protects the newborn against some disease such as infections disease, asthma and allergy. This effect may be due to the fact that breast milk acts as a mean for transporting many essential substances and useful and natural microflora that is dependent on diets of mothers. It is a complex emulsion which supplies not only the nutrients and energy required for the child's growth and development, but also factors which assist in microbiological protection, maturing and regulating the defense mechanisms including the immune system and accelerating postnatal maturation of the digestive system [1]. Only recently, it became accepted that breast milk constitutes a source of microorganisms that may colonize the infant gut and modulate its function [2]. Human breast milk includes several predominant bacterial species, such as

Staphylococci, *Streptococci*, *Micrococci*, *Lactobacilli*, *Enterococci*, *Lactococci* and *Bifidobacteria* [3, 4]. More recently, it has been shown that the human breast milk is considered to be an attractive source for probiotic strains. In 2003, two European research groups, one from Finland [5] and another from Spain [6] simultaneously and independently reported the isolation of lactic acid bacteria (LAB) from human breast milk. The probiotic potential of strains isolated from breast milk was evaluated and the authors concluded that it was similar, at least, to that of the strains commonly used in commercial probiotic products [7]. For instance, a Spanish dairy industry has recently industrialized one of these lactobacilli and launched into the market the first dried milk containing probiotic bacteria isolated from breast milk.

In the current study, we investigated the presence and identify of LAB in breast milk of healthy women that could be as a candidate possessing probiotic properties.

MATERIALS AND METHODS

Samples: Human breast milk samples were voluntarily donated by mothers from different locations in Sinai (Egypt). Mothers declared to be in good, healthy condition, having had normal and full-term pregnancy without infant or maternal perinatal problems. Thirty mothers provided 1 or 2 aseptically collected samples of breast milk. All samples were collected between days 1 and 10 postpartum. For sample collection, mothers were asked to carefully clean the mammary areola and breast skin with soap and rinse several times with sterile water. Mothers exerted slight pressure on their breast and the first 500 µl of breast milk were discarded, collecting the following 500-700µl in sterile tubes. Samples were immediately cooled to 5°C and transferred to the laboratory.

Isolation of Lactic Acid Bacteria: Breast milk was serially diluted in 0.1% peptone water and pour plate technique was used to isolate the organisms. Serial dilutions were plated onto Man-Rogosa-Sharp agar (MRSA) (pH 6.2 and 5.5) (Oxoid, UK), Trypticase Phytone Yeast (TPY) agar, (pH6.5) and MRS-cystein agar (pH 5.5) (Oxoid). Plates were incubated anaerobically at 37°C for 72h. The using of these mediums aimed to isolation of *Lactobacilli*, *Lactococci* and *Enterococci*. 1-3 colonies were randomly selected from each of duplicate MRSA, TPY and MRS-cystein agar plates of the highest dilutions showing growth colonies of *Lactobacilli* were subcultured in MRS broth and restreaked onto MRSA to ensure purity. *Lactococci* and *Enterococci* colonies were inoculated in M₁₇ broth (Oxoid). After growing the cultures were tested for purity on the suitable agar medium (M₁₇ or Kanamycin esculin azide agar base medium).

Identification of the Bacterial Strains: Isolates were tested for catalase activity [8], Gram-staining reaction [9] and cell morphology. All Gram positive and catalase negative rods were tested for growth in MRS broth at 15°C and 45°C and production of gas from glucose [10]. Gram positive cocci were identified according to Hardie [11]. The strains were tested for production of acids from carbohydrates and related compounds by use API (Montalieu, France) 20 STRP and API 50 CH strips and API CHL medium. The tests were done according to the instructions of the manufacturer and the results were read after incubation of strains at 37°C for 2 and 3 days.

Probiotic Properties of Lactic Acid Bacteria Strains: Antibacterial Activity: The antibacterial of the isolates was determined using cell-free neutralized supernatants

(CFNS). The CFNS were obtained from cultures grown in suitable broth medium (MRS or M₁₇ broth medium) for 18h at 37°C. Cultures were centrifuged (4000rpm for 15 min at 4°C) and the pH of the supernatant adjusted to 6.5-7.0 with 1M NaOH. The supernatant was then heated for 5 min at 100°C, cooled and stored at -20°C. Before use the CFNS were filter sterilized (0.2µm, Millipore). The neutralized supernatants were tested against *Escherichia coli* 0157:H7, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus cereus* and *Salmonella* spp., obtained from Dairy Microbiology Lab., National Research Centre, using agar well diffusion as described by Dabiza *et al.* [12]. All assays were performed in duplicate and the results presented are the means of duplicate trails.

Acid and Bile Salt Tolerance: The procedures of Tambekar and Bhutada [13] were used to determine the tolerance of various strains to bile and acid. Isolates were inoculated into suitable medium (MRS or M₁₇ of varying pH, *i.e.*, pH 2, 2.5 and 3, as well as broth with varying concentrations of bile salt (0.5, 1.0, 1.5 and 2%) and incubated at 37°C for 48h. Then 0.1 ml inoculums was transferred to MRS or M₁₇ agar by pour plate method and incubated at 37°C for 48h. The growth of LAB on MRS or M₁₇ agar plates was used to designate isolates as acid or bile salt tolerant.

Prebiotic Utilization: Three kinds of plants were investigated in this study: green bell pepper, artichoke leaves and green tea extracts. Fresh plants of green bell pepper and artichoke leaves were thoroughly washed then seeds were removed from green bell pepper, cut into smaller pieces and ground to mash using a kitchen blender after that it transferred to a clean dark bottles, sealed and stored at 4°C until it was used. Green tea was obtained from local market. All these prebiotics were solubilized in distilled water and sterilized by filtration using a 0.22 µm syringe filter unit (Millipore, Milan Italy). The strains were inoculated (1.5% inoculum) in 3-ml aliquots of MRS modified medium (Containing 10% each of three prebiotics) and grown anaerobically at 37°C for 21h. Utilization of prebiotics was evaluated by measuring the optical density at 600nm of each culture.

RESULTS AND DISCUSSION

Isolation and Identification of LAB Strains: In our study, fresh breast milk samples were obtained from 30 healthy mothers between days 1 and 10 postpartum. From these samples, 250 Lactic acid bacterial cultures were isolated. According to morphological and physiological

Table 1: Identification of *Lactobacillus* isolates according to phenotypical characteristics

Bacterial strain	% Total strains	Gram	Catalase reaction	Gas from glucose	Fermentation of carbohydrates								Growth at	
					Sucrose	Sorbitol	Maltose	Mannitol	Mannose	Rhamnose	Celluliose	Melibiose	15°C	45°C
<i>L. rhamnosus</i>	20.8	+	-	+	+	+	+	+	+	+	+	-	+	+
<i>L. plantarum</i>	13.2	+	-	-	+	+	+	+	+	-	+	+	+	-
<i>L. casei</i>	8.8	+	-	-	+	+	+	+	+	-	+	-	+	-
<i>L. fermentum</i>	8.8	+	-	+	+	ND	+	-	w	-	-	+	-	+
<i>L. acidophilus</i>	3.6	+	-	-	+	-	+	-	+	-	+	+	-	+

ND: no data available

w: weak positive reaction

*: % of strains over the total LAB isolates (250).

Table 2: Identification of cocci isolates according to phenotypical characteristics

Bacterial strain	% total strains*	Gram	Catalase reaction	Growth in			Growth at		CO ₂ production
				SF	NaCl 6.5%	pH 9.6	10°C	45°C	
<i>En. faecium</i>	18.4	+	-	+	+	+	±	+	-
<i>En. faecalis</i>	12.8	+	-	+	+	+	-	+	-
<i>Lc. lactis</i> ssp. <i>lactis</i>	8.8	+	-	-	-	-	+	-	-
<i>S. thermophilus</i>	4.8	+	-	-	-	-	-	+	-

*: % of strains over the total LAB isolates (250).

characteristics, the isolates were identified into five groups of rod (55.2%) and cocci (44.8%); lactobacilli, *Enterococcus*, *Lactococcus* and *Streptococcus thermophilus*. According to API50 CHL strips *L. rhamnosus* was the most frequently isolated strain (20.8%); followed by *L. plantarum* (13.2%) and *L. casei* and *L. fermentum* (each, 8.8%) and in a minor percentage, the strains of *L. acidophilus* for isolates belonged to the genus *Lactobacillus* (Table 1). Generally, our results confirmed the observation of Abrahamsson *et al.* [14] and Leila *et al.* [15] who reported that isolated bacteria from breast milk commonly included *L. rhamnosus*, *L. acidophilus*, *L. plantarum* and *L. fermentum*. The physiological characteristics important to the identification of cocci isolates to the species level are presented in Table 2. Based on these results and the schemes for identifying species developed by Hardie [11] and API 20 STREP, 46 strains were identified as *En. faecium*, 32 were identified as *En. faecalis*, 22 were identified as *Lc. lactis* ssp. *lactis* and 12 were identified as *S. thermophilus*. The most recent data available substantially confirmed the presence of *Enterococci* in breast milk. *En. faecium* and *En. faecalis* were found in breast milk [15, 16]. Other researchers isolated *En. faecium*, *En. faecalis*, *L. lactis* and *S. thermophilus* from human breast milk [2, 17]. The breast milk has been found to be a significant source of lactic acid bacteria that appear to be endogenous origin and not contaminants from breast skin [2]. Martin *et al.* and Solis *et al.* [17, 18]

reported the presence of cocci in human breast milk. The presence of lactobacilli in breast milk has been attributed to the existence of prebiotic oligosaccharides in this fluid [15, 19]. Out of 250 identified isolates, 18 isolates were recognized as probiotics on the basis of their acid and bile tolerance and antibacterial activity. The probiotics were *L. rhamnosus*, *L. plantarum* (each, 4 strains), *L. casei*, *L. fermentum* (each, 2 strains), *L. acidophilus* (1 strain), *En. faecium* (3 strains) and *Lc. lactis* (2 strains).

Probiotic Properties

Acid and Bile Salt Tolerance: Probiotics potential of LAB is necessarily its ability to resist bile salts and acidic pH [20]. In this study, 13 *Lactobacillus* isolates and 5 cocci isolates showed acid tolerance at pH 2 and bile salt tolerance at 2%. Before reaching the intestinal tract, probiotic bacteria must first survive transit through the stomach where the pH can be as low as 1.5 to 2 [21]. Tolerance to bile salts is a prerequisite for colonization and metabolic activity of bacteria in the small intestine of the host [22]. This will help *Lactobacilli*, *Enterococci* and *Lactococci* to reach the intestine and colon and contribute in balancing the intestinal microflora [13].

Antibacterial Activity: 77.2% of the tested strains didn't display an inhibitory activity, while only 57 strains, belonging to different tested species, were active against some of the target organisms (Table 3).

Table 3: Antibacterial activity of isolated Lactic acid bacteria against pathogenic and spoilage bacteria

Indicator strains	Inhibitory activity																	
	<i>L. rhamnosus</i>		<i>L. plantarum</i>		<i>L. casei</i>		<i>L. fermentum</i>		<i>L. acidophilus</i>		<i>En. faecium</i>		<i>En. faecalis</i>		<i>Lc. lactis</i> ssp. <i>lactis</i>		<i>S. thermophilus</i>	
	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
<i>E. coli</i> 0157:H7	14	38	4	29	2	20	2	20	1	8	13	33	1	31	2	20	0	12
<i>S. aureus</i>	14	38	7	26	19	3	4	18	3	6	10	36	0	32	0	22	0	12
<i>Ps. aeruginosa</i>	4	48	4	29	7	15	3	19	1	8	3	43	2	30	2	20	1	11
<i>B. cereus</i>	5	47	6	27	14	8	10	12	1	8	0	46	0	32	11	11	3	9
<i>Salmonella</i> sp.	6	46	9	24	2	20	2	20	2	7	3	43	3	29	2	20	1	11

(+): number of positive strains (strains that have antibacterial activity).

(-): number of negative strains (strains that have not antibacterial activity).

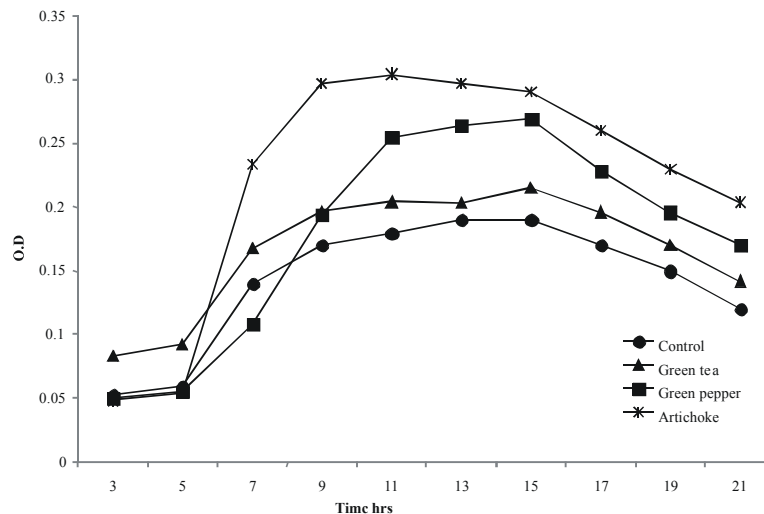


Fig. 1: The effect of prebiotic substances on the growth curve (O.D at 600 nm) of *L. plantarum* at 37 oC for 21 hrs

Similar results have been reported by Jara *et al.* [1] and Heikkila and Saris [5]. The spectra of inhibition varied among the species and *L. rhamnosus*, *L. casei* and *L. plantarum* were the most effective. *S. aureus* was inhibited by 14, 19 and 7 strains of *L. rhamnosus*, *L. casei* and *L. plantarum*, respectively. This confirms the results obtained by Dabiza *et al.* [12] who isolated strains from dairy products, effective against this target organism. Inhibitory activity was demonstrated by some of all the test strains against *E. coli* 0157:H7, except for *S. thermophilus*. These findings are in agreement with that reported by Ibhanebhor and Otobo [23] for inhibitory activities of human colostrum against *S. aureus* and coliform organisms. This may be due mainly to the high immunoglobulins (Igs) content of colostrum [24]. Also, Abd El-Salam and Al Rubayyi [25] found that the breast milk exerted bactericidal activity against *E. coli*, *Ps. aeruginosa* and *S. aureus*. *Salmonella* sp. inhibition halos were evident with some of all the lactic cultures. Inhibition of other

bacteria was variable according to the test strain. Additionally, all stains of *S. thermophilus* displayed a narrow range of antimicrobial activity, whereas the strains of *En. faecium* inhibited a wider range of indicator bacteria, including *E. coli* 0157:H7, *S. aureus*, *Ps. aeruginosa* and *Salmonella* spp. (*Enterococcus* spp. has been reported to produce bacteriocins which inhibited Gram-positive food-borne bacteria and intestinal pathogens [13, 26]. Human breast milk constitutes an interesting source to obtain new specific probiotic stains for neonates aiming at assisting a proper development of the gut microbiota and the immune development in infants who, for different reasons, can't be breast-fed.

The breast milk collected in this study contained strains of *L. rhamnosus*, *L. plantarum*, *L. casei*, *L. fermentum*, *L. acidophilus*, *En. faecium*, *L. fermentum*, *Lc. lactis* sp. *Lactis* and *S. thermophilus*. Therefore, breast milk is considering a significant source of lactic acid bacteria that appear to be of endogenous origin [2].

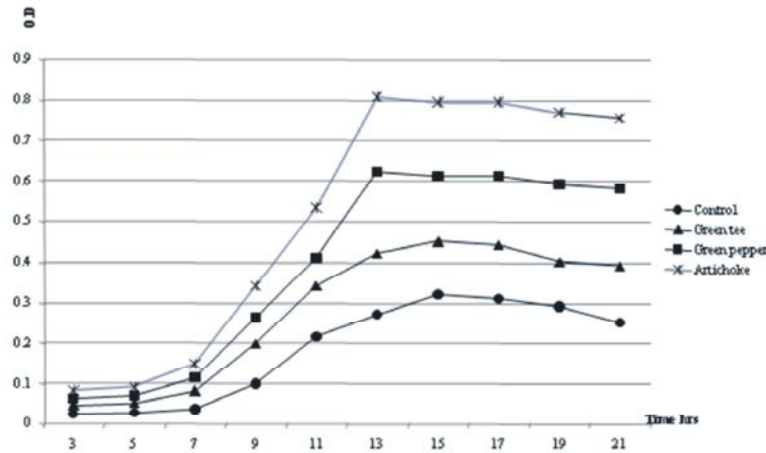


Fig. 2: The effect of prebiotic substances on the growth curve (O.D at 600 nm) of *L. shammasar* at 37°C for 21 hrs

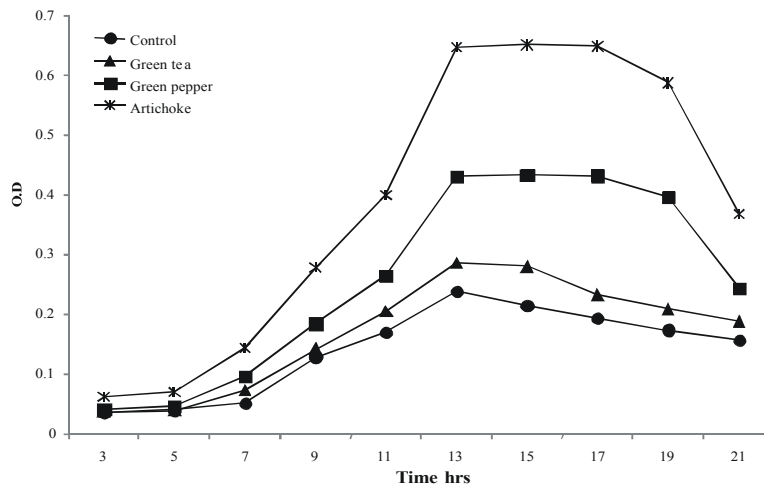


Fig. 3: The effect of prebiotic substances on the growth curve (O.D at 600 nm) of *Lactococcus lactis* at 37°C for 21 hrs

Since breast milk has been suggested as vehicle for potentially probiotic LAB, it could be considered as a natural synbiotic food that is a mixture of probiotics and prebiotics. It has also been proved that breast feeding beneficially can affect infants by improving the survival and implantation of live dietary microorganisms in gastrointestinal tract [15, 27].

Prebiotic Utilization: All the tested strains showed the highest cell growth with artichokes (Figs. 1, 2 and 3). Green bell pepper was also fermented by all the strains but at a lower rate. Green tea was poorly fermented. These results demonstrated that artichokes are a suitable food carrier that allows the survival of potentially probiotic strains as previously shown by Valerio *et al.* [28]. The high cell viability shown can also be explained by the roughness of the vegetable structure, which may offer protection to the bacterial population. In general, all tested prebiotics were effective

in enhancing growth rate of all strains than control treatment. This may be due to the composition of these plants which contain complex carbohydrate and dietary fibers.

CONCLUSION

In conclusion, the breast milk could be a good and safe source for isolation of probiotic bacteria and for improve intestinal microflora of infants. Study will affirm their use in the development of new pharmaceutical preparations and functional foods that contain milk probiotics for the betterment of health of the public.

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