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Isolation of Lactic Acid Bacteria from Ewe Milk, Traditional Yoghurt and Sour Buttermilk in Iran

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Authors' contributions

This work was carried out in collaboration between all authors. MI designed the study, MA performed the statistical analysis, MI, HE, NM wrote the protocol, HE, MI wrote the first draft of the manuscript. MAKT, HE, NM managed the analyses of the study. MI, HE, NM managed the literature searches. MM managed the sampling. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

A total of 63 samples including ewe milk, yoghurt and traditional buttermilk were collected from Myaneh and Hashrood (Azarbayjan-e-Sharqi, Iran) and screened for the presence of Lactic Acid Bacteria (LAB). Based on routine cultural characteristic, general morphological and biochemical assay, 77 out of 168 bacterial isolates were identified as LAB. These isolates were examined for the presence of inhibitory activity against other randomly selected LAB isolates. Thirty-three strains showed antagonistic activity against the closely related LAB strains and were further challenged against other gram-positive and gram-negative pathogens including *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella entritidis*. Based on their zones of inhibition diameters the isolates showing maximum inhibitory activity against these pathogens were selected for detailed

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investigations. The selected isolates were identified to species level by 50CHL API system and were challenged to heat, acid and bile salt. Most of the strains were able to survive at different pH ranges, while one strain of *Pedicoccus acidilactici* and *Lactobacillus paracasei* were able to tolerate all ranges of pH during 24 h of incubation. In addition, *Lactobacillus brevis* was found as the most resistant strain being able to resist all concentrates of bile after 4 h. The results indicated the probiotic potential of the isolates, as majority of the selected LAB isolates were capable of resisting high temperatures, acidic pH values and bile concentrations of 0.7%.

Keywords: Traditional buttermilk; lactic acid bacteria; antimicrobial activity; probiotic bacteria.

1. INTRODUCTION

A number of reports have emphasized the significance of food fermentation mainly because of degradation or inactivation of anti-nutritive factors, toxins, as well as improvement of digestibility of foods that leads their major role in the diet of different regions (Mathara et al., 2008). Although, different components of milk are involved in fermentation process, LAB commonly metabolize lactose into lactic acid resulting in pH reduction and higher titratable acidity (TA) and creating an environment that is unfavorable to pathogens and spoilage organisms (Aslim et al., 2005).

According to reports, it appears that Middle East is the origin of fermented dairy products mainly yoghurt (Tamime and Robinson, 2007). In Iran, a number of traditional dairy products are consumed of which yoghurt, well known, as Mast is one of the most popular fermented milk products. While, traditionally made sour buttermilk especially made from ewe milk is more common in rural areas of the country.

Several previously published reports have indicated the presence of *Lactobacillus* strains in sheep and cow milk (Mobarez et al., 2008). In addition, several studies have shown the inhibitory activities of numbers of LAB such as *Lactobacillus brevis* isolated from Turkish dairy products (Aslim et al., 2005) and *Lactobacillus acidophilus* isolated from Iranian yoghurt against *Staphylococcus aureus* (Mobarez et al., 2008).

It is a well-established fact that the composition of LAB in these traditional dairy products is varied and inconstant. Owing to the said health benefit of buttermilk in the rural areas it appeared interesting to evaluate the microbial load of these products with specific emphasis on probiotic bacteria mainly LAB. The main objective of present study was to investigate LAB of traditional sour buttermilk made from ewe's milk, which might provide important information regarding its probiotic potential and its utilization in the future.

2. MATERIALS AND METHODS

2.1 Bacterial Strains and Culture Conditions

All LAB strains used in this study were grown in MRS broth (HiMedia India) at 37ºC for 24-48 h in anaerobic jars. All pathogenic strains used in this study were grown in BHI (HiMedia-India) at 37ºC for 18-24 h under aerobic condition.

All strains were maintained at 4˚C (in aerobic condition) and renewed every week for short-term preservation. The long-term conservation of the purified isolates was carried out in MRS broth with sterile glycerol (15%) and stored at -70ºC (Badis et al., 2004).

2.2 Collection of Ewe Milk and Preparation of Buttermilk

Ewe milk samples were collected from 30 sheep herds in Myaneh (15 herds) and Hashrood (15 herds) cities in north-west of Iran. All samples were collected according to ISO 707 in sterile bottles of 250 mL and transported to the laboratory under refrigeration (4ºC) within 36 h (AOAC, 2002).

The traditionally made yoghurt and sour buttermilk samples were also collected from the same area. Fig.1 shows the preparation operation of yoghurt and sour buttermilk. As indicated, the samples are prepared in a traditional device known as Mashk (Fig. 2) that is made from hide (sheepskin) and is used for making butter and buttermilk from yoghurt. This device is also used widely for preservation of fermented dairy products for 10 to 20 days at temperatures not exceeding 20ºC.

2.3 Isolation and Identification of LAB

One ml of the collected samples (ewe milk, yoghurt and sour buttermilk) were inoculated in MRS broth and incubated at mentioned conditions until the appearance of growth. A loop full of the grown culture broth was then plated on MRS agar plates and pure colonies selected after incubation and tested for Gram identification, cell morphology and catalase activity (Karimi Torshizi et al., 2008).

2.4 Screening for Antagonistic Interactions among LAB Strains

The antagonistic effects of isolated LAB against four other selected LAB isolates (which showed the fastest growing strains and retained their maximum viability during storage at refrigerated temperatures) were determined by using an agar diffusion method (Aslim et al., 2005) with slight modifications. The freshly grown overnight cultures of the producer and indicator strains were adjusted to McFarland Index 3 prior to use. The surface of MRS agar plates were evenly streaked with 0.1 ml of a 24h broth culture of the selected indicator strains, with a sterile cotton swab. The culture broth of the producer strains (150ul) were poured into the wells (10mm) made in these agar plates with a sterile borer (2.5mm). All plates were stored for 2h at 4ºC prior to incubation at 37ºC for 24h. The antimicrobial activity was recorded as appearance of clear zone around the wells and the zone diameter (+:3mm<zone, ++:3mm<zone<5mm, +++:5mm<zone<7mm) measured in millimeter. All tests were run in duplicate.

2.5 Antimicrobial Activity against Pathogens

Thirty-three LAB isolates demonstrating high antibacterial activity against other selected LAB isolates were further checked against other Gram positive and negative pathogens by agar well diffusion method described earlier. *Staphylococcus aureus* (PTCC 1112), *Listeria monocytogenes* (PTCC 1298), and *Salmonella enteritidis* (local isolate) were used as indicator culture. As mentioned earlier, the culture broths of both the producer and indicator strains were adjusted to McFarland Index 3 prior to use.

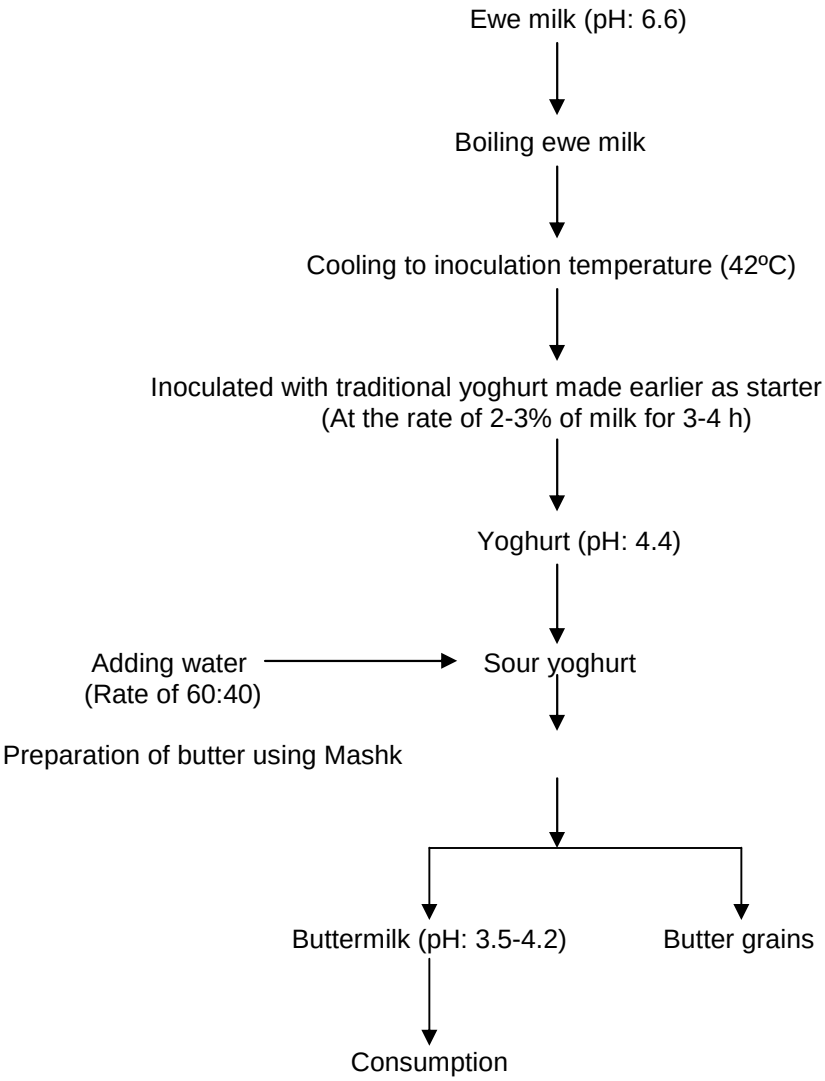

Fig. 1. Preparation of traditional yoghurt and sour buttermilk.

2.6 Identification of Isolated LAB to Species Level

The 10 LAB strains showing maximum activity against the tested pathogens were identified to species level and their carbohydrate fermentation profiles were investigated using API 50 CHL medium (Bio-Meriux, France) according to the manufacture's instruction (Yukeskdag et al., 2004).

2.7 Heat Tolerance

Heat resistance of identified LAB strains was tested by exposing the overnight grown cultures to 55ºC and 80ºC for 3 min according to the method described earlier (Christiansen

et al., 2006). The growth was recorded by inoculating 100ul of the treated cultures on MRS agar plates.

2.8 Acid Tolerance

Acid tolerance of the selected LAB isolates was determined by the method described by Liong and Shah (2005), with slight modifications. The pH of the MRS broth were adjusted to 2.0, 3.0, 4.0, 5.0, and 6.0 by adding 1N HCl. Overnight grown culture broths of the selected LAB isolates were centrifuged and pellet collected, washed twice with PBS and adjusted to Mcfarland 3 Index. Approximately 100ul of the cell culture was inoculated in these tubes and their growth recorded after 0, 1, 2, 4, 24 h of incubation at 37ºC. The cell count (cfu/ml) at 0 h was considered as control.

2.9 Bile Tolerance

The effect of bile acid tolerance on the growth of identified LAB strains was determined according to the method described by Gilliland (1984) with slight modifications. The pH of all MRS broth was adjusted to 6.5 and then, 20ul of freshly grown culture of each strains were added to MRS broth with different levels (0.1, 0.3, 0.5, .07, 1, 2, 3 % w/v) of bile salts (oxgall). The bacterial growth monitored after 0, 2, 4 and 24 h of incubation at 37ºC by colony count. The cell count (cfu/ml) at 0 h was considered as control.

3. RESULTS

Seventy-seven out of 168 bacterial colonies from 63 samples of ewe milk, yoghurt and sour buttermilk (Table 1) were identified as belonging to genus LAB based on their gram reaction, morphology and catalase test. All isolates were catalase negative and Gram-positive cocci (n=30), bacilli (n=38) and cocobacilli (n=9).

3.1 Antagonistic Reaction

3.1.1 Antagonistic interaction against four selected LAB

All of the isolated LAB strains exhibited antibacterial activity when exposed to other selected LAB isolates used as indicator strain. According to the results, majority of the bacteria isolated from milk or yoghurt exhibited activity against indicator bacteria that were isolated from the same products. For instance, most of the bacteria isolated from milk had activity against indicator bacteria isolated from milk. We also observed that most of bacilli appearing isolates exhibited higher antibacterial activity compared to cocci appearing isolates.

3.1.2 Antagonistic activity against pathogens

Among 77 LAB isolates, only those exhibiting highest activity based on their zone of inhibition diameters (ZID) were further screened for their antagonistic activity against other gram-positive and gram-negative pathogens. The selected LAB isolates screened in this study were isolated from milk (24), yoghurt (1) and buttermilk (8) samples. According to their ZID all isolates showed different level of activity against the tested pathogens.

Table 1. Morphological, cultural and physiological characteristics of the LAB isolates

Group	Gram +ve cocci			Gram +ve bacilli			Gram +ve coccobacilli		
	Milk	**Yoghurt**	**Buttermilk**	**Milk**	**Yoghurt**	**Buttermilk**	**Milk**	**Yoghurt**	**Buttermilk**
No. of isolates	24	2	4	4	16	18	8	-	1
Spore forming	-	-	-	-	-	-	-	-	-
Catalase test	-	-	-	-	-	-	-	-	-

Table 2 shows that among the tested isolates, twenty-five inhibited the growth of *Salmonella enteritidis* and twenty isolates inhibited *Staphylococcus aureus* and only ten inhibited the growth of *Listeria monocytogenes*. According to results, eight of the 33 LAB isolates could not inhibit the growth of any of the pathogen tested while, 5 LAB isolates inhibited 1 of the pathogens and 10 inhibited 2 of the pathogens. All ten isolates exhibiting antilisterial activity were also inhibitory towards other two pathogens used in study and were thus selected for further detailed investigations.

Table 2. Antimicrobial activity of LAB isolates against other pathogenic strains

No	Isolates	*L. monocytogenes*	*Staph. aureus*	*S. enteritidis*
1	M2	-	+	+
2	M3	++	+++	++
3	M4	-	-	+
4	M5	-	+	+
5	M7	-	-	-
6	M8	-	-	-
7	M9	-	-	-
8	M10	-	-	-
9	M11	+++	++	+++
10	M15	-	-	+
11	M16	-	++	++
12	M17	-	-	+
13	M20	++	++	+
14	M21	+	+	++
15	M22	-	+	+
16	M23	+	++	++
17	M25	-	+	+
18	M26	-	_	+
19	M27	-	+	+
20	M29	+	+++	+
21	M30	++	+	+
22	M33	-	+	+
23	M35	-	+	+
24	M36	+	++	++
25	Y53	-	+	+
26	B.M64	-	-	+++
27	B.M68	-	-	-
28	B.M71	-	+	+
29	B.M73	+++	+	++
30	B.M74	-	-	-
31	B.M75	-	-	-
32	B.M76	-	-	-
33	B.M77	+	+	++

-: no zone of inhibition.
+: 3mm< zone.
++: 3mm< zone <5mm.
+++: 5mm< zone <7mm.
M denotes bacterial isolates from milk while Y and B.M are LAB isolates from yoghurt and buttermilk, respectively.

The selected ten isolates were identified to species level (Table 3) as *Lactobacillus pentosus*, *Lactobacillus paracasei* (two strains), *Lactobacillus brevis*, *Pediococcus acidilactici* (four strains) and *Lactococcus lactis* (two strains).

Table 3. Identification of the selected LAB isolates to species level using standard API 50CH identification kit

No	Sample	Source	Species identified
1	M3	Milk	*Lactobacillus pentosus*
2	M11	Milk	*Pediococcus acidilactici*
3	M20	Milk	*Lactococcus lactis*
4	M21	Milk	*Lactobacillus paracasei*
5	M23	Milk	*Lactococcus lactis*
6	M29	Milk	*Lactobacillus paracasei*
7	M30	Milk	*Lactobacillus brevis*
8	M36	Milk	*Pediococcus acidilactici*
9	BM73	Buttermilk	*Pediococcus acidilactici*
10	BM77	Buttermilk	*Pediococcus acidilactici*

3.2 Resistance to Heat, Acid and Bile

All the selected LAB isolates in study were able to resist 55 and 80ºC for 3 min. However, the level of heat resistance differed among the isolates as was evident by their cfu/ml recorded at different time intervals (data not shown).

The effect of acid on the viability of the selected LAB isolates after 1, 4 and 24 h is shown in Table 4. Among all the isolates in study, *L. paracasei* M29, *P. acidilactici* BM77 were the most acid tolerant as they were able to resist acidic pH values of 2.0 even after 24h of incubation. While, *L. lactis* (M20) appeared to be most acid sensitive strains as could not resist pH values ranging from 2 to 5. In addition, two of the isolates namely *L. brevis* (M30) and *L. pentosus* (M3) were not able to grow only at pH value of 2.0, but good growth was observed at higher pH values used in this study. All LAB isolates were able to tolerate pH 6.0.

As shown in Fig. 4, all strains showed variable range of resistance to different concentrations of bile salts during 4 h of incubation. *P. acidilactici* (M36) and *L. lactis* (M20) appeared to be highly bile sensitive as were not able to grow in bile salt concentrations of 0.5% and 0.7% within 4h of incubation, respectively. While, the most resistant strains in this study appeared to be *L. brevis* M30 which showed highest growth in 1% bile after 4 h and *P. acidilactici* BM73 which was also able to resist 0.3% bile even after 24 h. The growth appeared reciprocal to bile salt concentrations, and with an increase in bile concentration, the growth decreased significantly in all the tested LAB isolates.

Table 4. Acid tolerance of selected LAB isolates during different time intervals

No	LAB isolates	pH														
		2.0			3.0			4.0			5.0			6.0		
		1h	4 h	24 h	1h	4 h	24 h	1h	4 h	24 h	1h	4 h	24 h	1h	4 h	24 h
1	M3	-	-	-	+	+	-	+	+	-	+	+	+	+	+	+
2	M11	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+
3	M20	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
4	M21	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+
5	M23	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
6	M29	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	M30	-	-	-	+	+	-	+	+	-	+	+	-	+	+	+
8	M36	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+
9	BM73	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+
10	BM77	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+: growth observed
-: No growth observed.

4. DISCUSSION

The traditional fermented dairy products can be possibly a good source of potential probiotic organisms. In Iran, a number of researchers have reported the isolation of LAB from traditional dairy products like doogh (a very popular minted yogurt beverage made by yoghurt and water seasoned with mint and salt), butter, kashk (a thick whitish liquid similar to whey) and cheese (Tajabadi Ebrahimi et al., 2011). However, there are some traditional fermented dairy products in Iran, which have yet not been evaluated for their health benefit, mainly their probiotic properties. Among such fermented dairy products, one is buttermilk, the microbial ecology and beneficial health effects of which has not been reported earlier. These products are famous especially in the rural areas because of their good natural tastes and flavors.

Our results showed that most of the strains isolated from ewe's milk were gram-positive cocci, while bacilli were more dominant in yoghurt and sour buttermilk samples. It seems that the abundance of bacilli in yoghurt and sour buttermilk is due to the starter cultures such as *L. bulgaricus* and *Strep. thermophilus*, which are mainly responsible for yoghurt formation, as well as higher viability of *L. bulgaricus* in acidic environment (Tamime and Robinson, 2007). Although, the optimal pH for growth of *Lactococci* ranges from 6 to 6.5 (Marth and Steele, 2001) but in our studies the pH of sour buttermilk was approximately 3.8 (Mohamadi et al., 2009), and a number of LAB bacteria were viable at this pH value.

As a functional probiotic, anti-pathogen activity is one of the important properties to be considered. Antimicrobial activity of selected strains may be due to the combination of factors including acid, H_2O_2 and bacteriocin like substances (Gilliland et al., 1984). The 10 selected LAB isolates showing higher inhibition against closely related strains were further tested against other pathogens such as *Salmonella enteritidis*, *Staph. aureus*, and *L. monocytogenes*. The pathogens listed have been reported to be of importance in food products (Gurira and Buys, 2005). During our investigations, we observed that majority of the selected LAB strains were able to inhibit the growth of *Salmonella enteritidis* compared to *Staph. aureus* and *L. monocytogenes*. While, ten selected strains were able to inhibit the growth of all three pathogens used in study. In addition, due to high concerns about the presence of *Listeria* in dairy products, especially as this pathogen has the ability to withstand a large variety of environmental conditions such as refrigeration temperatures, only the isolates demonstrating antilisterial activity were selected for further investigations.

Moreover, viability and survival of probiotic bacteria during passage through the stomach is an important parameter to reach the intestine and provide beneficial effect (Chou and Weimer, 1998). According to reports, the pH in stomach is 0.9, with the presence of food the pH increased and reaches up to 3.0. After the ingestion, 2-4 h takes the stomach to become empty (Erkkila and Petaja, 2000). However, some factors like buffering capacity of food, which is a major factor affecting pH, rate of gastric emptying and physiological state of the bacterium, have affect on the survival strains in stomach (Bertazzoni Minelli et al., 2004). In our studies, some of the tested isolates resisted acidic pH values of 2 even for 24 h. The acid resistance of the selected isolates might be exploited for their use as a probiotic as they might withstand gastric stress and survive in high acid food for longer periods without large reduction in numbers.

The next challenge for potential probiotic survivors in gastrointestinal tract is exposure to bile salts in the upper part of the small intestine (Chou and Weimer, 1999). The concentration of human bile ranges from 0.3% to 0.5%, however 0.3% is considered critical concentration for

screening for resistant strains (Gilliland et al., 1984). The most resistant strains in this study appeared to be *P. acidilactici* BM73 that not only resisted 1% bile salt for 4 h but also was also able to survive in 0.3% bile for 24 h. According to our results, *P. acidilactici* BM73, *L. lactis* M23 and *L. paracasei* M29 appeared superior to other strains used in study, respectively. These three isolates were also the most acid tolerant strains, while *L. brevis* M30 also being highly bile tolerant was not able to resist acidic pH values. The difference in acid and bile tolerance of one strain from two species within same genus may be due to differences in their cell wall structure (Conway et al., 1987).

Apart from being acid and bile resistant, another important property of some of these isolates was their temperature tolerance. Heat resistance of bacteria is affected by genetic differences among species, the physiological status of the cells and environmental factors such as pH, water activity, salt content and preservatives (De Angelis et al., 2003). It has been reported previously that some strains of *Lactobacillus* can grow at low and high temperatures (below 15ºC and some strains up to 55 ºC), while the thermal death point of *P. acidilactici* has been reported to be 70ºC for 10 min according to Bergey's Manual. Majority of LAB isolates in study were able to resist 80ºC for 3 minutes but their growth rate differed significantly. At 55ºC, all isolates survived within the mentioned time period without any significant effect on their growth rate. It is obvious that the high temperature used for heating milk (boiling) and adding cold water to sour yoghurt, results in an increased bacilli count in sour yoghurt and buttermilk (Tamime and Robinson, 2007). Interestingly, our results showed that all selected strains tolerate 88ºC for 3 min and because we selected these isolated after boiling which is shown in Fig. 1. Therefore, these selected strains might pass boiling process also, it could be possible that these strains lived in the Mashk and added to sour buttermilk after boiling process.

All ten selected strains are considered suitable for qualified presumption of safety (QPS) (EFSA2007b). In addition, these products have consumed for many years especially in rural areas of the country.

Fig. 2. Mashk

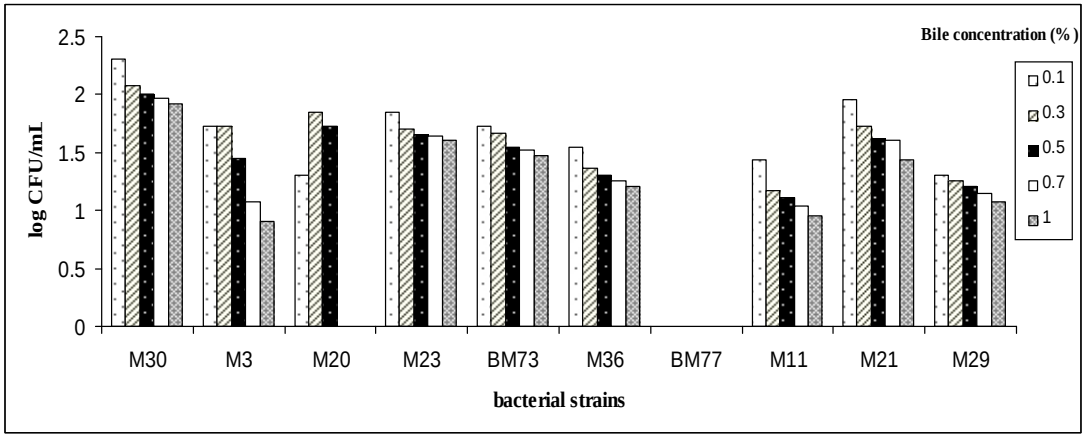

Fig. 3. Bile tolerance of selected LAB isolates as a control (time 0)

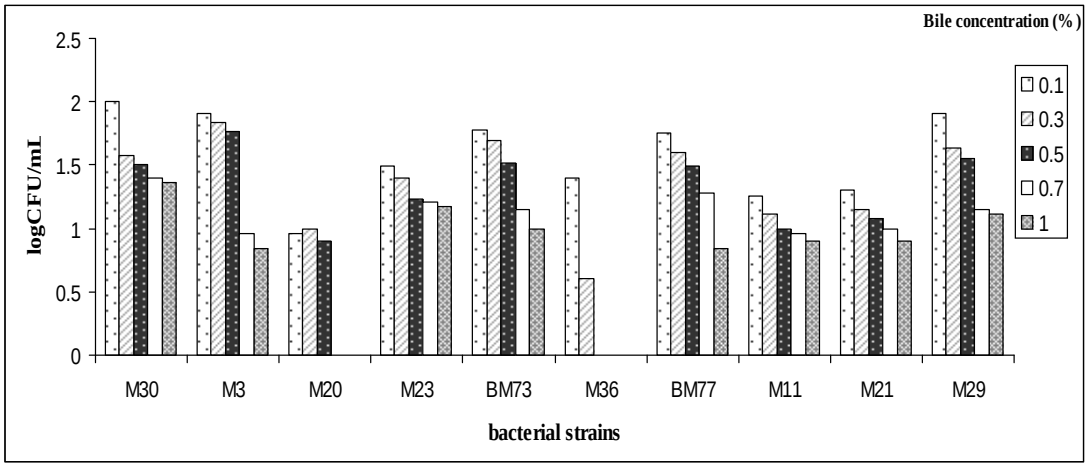

Fig. 4. Bile tolerance of selected LAB isolates after 4h

5. CONCLUSION

According to our knowledge, this is first report that states the presence of LAB strains in raw ewe milk, yoghurt and traditional sour buttermilk in Iran. This study proved the presence of viable non-starter LAB micro flora in these products. Among selected isolates, we found *Pediococcus acidilactici* in both milk and buttermilk, which indicates that these isolates were able to tolerate high processing temperatures and survive in final product (buttermilk). Furthermore, this strain might be added to product after boiling which comes from Mashk. The antagonistic activity possessed by these isolates might be used for the control of unwanted pathogens mainly in dairy products. Overall results of this research suggests that the selected LAB strains isolated might be appear to possess probiotic potential, and hence could be exploited further for their use in fermented dairy products

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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