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Technological Characteristics of *Lactobacillus* spp. Isolated from Iranian Raw
Milk Motal Cheese

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19

20 **Abstract**

21 Lactobacilli produce large amounts of bioactive metabolites, which makes them suitable for
22 numerous technological applications. The objective was to evaluate the technological
23 characteristics of 16 *Lactobacillus* isolates from Iranian raw milk Motal cheese, including
24 acidifying ability, production of extracellular polysaccharides (EPS), gamma-amino butyric acid,
25 proteolytic, lipolytic, autolytic, and amylolytic activity, resistance to acid conditions as well as
26 biogenic amine production and susceptibility to antibiotics. Most of the examined strains
27 presented high proteolytic and autolytic activity. Low acidifying ability was observed for all
28 isolates, except for *L. plantarum* (M19) which reduced pH to 4.6 after 12h. The highest
29 proteolytic activity (30-mm clear halo) was observed for *L. brevis* (M4). Lipolytic activity was
30 never observed and most of the isolates did not produce biogenic amines. The highest percentage
31 of antibiotic resistance (87%) was recorded for kanamycin. All isolates were sensitive to
32 chloramphenicol except *L. brevis* (M11). EPS production was detected in all strains, most strains
33 (90%) produced mucoid EPS and 10% secreted EPS with a “ropy” character. Acid resistance
34 showed all isolates were able to grow at pH 3.0 and 4.0. *L. brevis* (M4) presented the best
35 technological characteristics and is proposed for inclusion as co-culture in the production of
36 Motal Cheese.

37 **Keywords:** Motal Cheese, *Lactobacillus brevis*, *Lactobacillus plantarum*, Starter Cultures,
38 Adjunct Cultures

39 **1. Introduction**

40 Cheese is considered one of the most important fermented milk products in the world, and thus
41 traditional cheeses have been widely studied (Tavaria *et al.*, 2003; Ahmadova *et al.*, 2013; Nieto-
42 Arribas *et al.*, 2010). Cheeses and yogurts, along with other fermented dairy products have been
43 locally produced in large quantities for centuries in different parts of Iran (Azizi *et al.*, 2017,
44 Edalatian *et al.*, 2012; Farimani *et al.*, 2016; Shakibaie *et al.*, 2017). Motal cheese is one of the
45 oldest local raw goat's or sheep's milk cheeses produced in the region known as "Dasht-e-
46 Moghan". Motal cheese has an oily, brittle, and granular texture. Ripening takes place in a bag
47 made of sheep's or goat's skin. The mature cheese has an intense and distinct taste somewhat
48 similar to the French Roquefort cheese, which may be due to its high fat and salt content
49 (Ahmadova *et al.*, 2013; Azizi *et al.*, 2017).

50 Lactic acid bacteria (LAB) can be found in a wide range of natural environments and are
51 considered to be the dominant microbiota in milk and dairy products (Ayad *et al.*, 2004). Some
52 characteristics of LAB, such as acid production, probiotic, proteolysis, lipolysis and autolysis
53 have contributed to their use as starters, and are investigated when selecting these bacteria and
54 studying their characteristics (Piraino *et al.*, 2008; Mantzourani *et al.*, 2019). Native strains of
55 LAB are of interest to researchers and dairy producers due to their inherent resistance to phages,
56 their antimicrobial properties, and their ability to produce the typical taste and smell during the
57 production of various types of cheeses. Additionally, the general implication that current
58 hygienic standards of the dairy industry like pasteurization and non-thermal processes such as
59 microfiltration, bacto-fugation proved milk of excellent microbiological quality. These
60 technologies enhance the safety of dairy products, also lead to a noticeable reduction of the
61 autochthonous microbiota, like nonstarter lactic acid bacteria (NSLAB), that transmit diverse and
62 typical characteristics to cheeses (Ayad *et al.*, 2004; Milesi *et al.*, 2010).

Lactobacilli are dominant species in raw milk cheeses since these organisms can continue to grow under the adverse conditions of ripening. Also, due to their excellent proteolytic activity LAB help create the original organoleptic properties of the final product (Torres-Llanez *et al.*, 2006). Local and starter-free raw milk cheeses harbor an impressive diversity of LAB strains which can be harnessed to supplant or supplement current starters and/or adjunct cultures (Kelly and Ward 2002; Rademaker *et al.*, 2006; Brandsma *et al.*, 2008; Alegría *et al.*, 2016).

In order to preserve the originality of traditional cheeses under quality labels, new strains are highly demanded. Native strains utilization would guarantee the safety of the product and produce cheese similar to the traditional cheese characteristics (Florez *et al.*, 2006; Alegría *et al.*, 2009). The different enzymatic activities of indigenous strains during ripening, including proteolytic, lipolytic, and autolytic activity, have been used as criteria to select *Lactobacillus* species and strains as adjunct cultures for dairy (Hannon *et al.*, 2003; Kiernan, Beresford, O’Cuinn, & Jordan, 2000; Ahmadova *et al.*, 2013). Autolytic activity is of particular interest because it let the key intracellular enzymes to reach their substrate more easily. Resistance to antibiotics to evaluate the performance and safety of isolates should be taken to consideration when selecting as starter culture.

In this study, 16 native *Lactobacillus* strains extracted from traditional Motal cheeses were evaluated for several technologically-relevant properties to select suitable co-culture components for the industrial production of Motal cheese and, if appropriate, other biotechnological applications.

2. Material and Methods

2.1. Bacterial strains and culture conditions

Azizi *et al.* (2017) studied and genotyped 16 *Lactobacillus* species in Motal cheese. Of the species, 11 belonged to *L. brevis*, 4 to *L. plantarum*, and one to *L. casei*. Stock cultures were frozen at -80°C in MRS broth (Quelab, Canada) with 20% (v/v) glycerol (Table 1). Briefly, isolation and phenotypic identification of lactobacilli was conducted following some biochemical reactions and implementation the gram reaction and catalase activity on isolates (Azizi *et al.*, 2017). All gram-positive and catalase-negative isolates were chosen for further analysis: DNA extraction and molecular identification. DNA extraction was performed with the GenElute™ Bacterial Genomic DNA kit (Thermo Fisher Scientific, Germany) according to the manufacturer's protocol. Primers 27F (5'-AGAGTTTGATYMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3) were used for amplification of the gene 16S rRNA (Alegría *et al.* 2009). Sequencing results were compared with the databases using the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Isolates with 98% or higher similarity in sequences were identified as the same species (Stackebrandt and Goebel 1994; Palys *et al.* 1997).

Table 1. here.

2.2. Physiological properties

In order to evaluate the effect of different temperatures on growth, isolates were first incubated in a water bath at 25, 30, 37, 40 and 45°C for 24 h. After incubation, the optical density of the cultures was measured at 600 nm (OD₆₀₀) by a spectrophotometer according to the method of Sanchart *et al.* (2016). In summary, lactobacillus strains were kept in a water bath for 24 hours at 25, 30, 37, 40 and 45°C. Strains were then incubated at 25, 30, 37, 40 and 45 for another 24 hours. Spectrophotometer measurements were done in OD₆₀₀ (600 nm) in the first and last 12 hours of incubation. Turbidity formation, could be as a result of growth.

Technological characteristics

2.2.1. Acidifying ability

To reactivate isolates, bacteria were introduced into MRS broth and kept at 37° overnight. 10 ml of sterile skimmed milk (RSM 10%, w/v) (Merck, New Zealand) was inoculated (1%, v/v) with reactivated bacteria and stored at 37 °C. pH was read with a pH-meter after 24 h of incubation and values were recorded as Δ pH (Garriga et al., 1996).

2.2.2. Acid tolerance test

Isolated strains were allowed to proliferate in MRS broth at 37°C for 24 h. Ten ml of the liquid culture was then poured into tubes and was centrifuged at $2000 \times g$ for 10 min at 4°C. The resulting pellets were rinsed twice with sterile PBS (pH 7.2; Sigma-Aldrich) and suspended in 1 ml of PBS. For each strain, 0.1 ml of the culture suspension (with approximately 1.5×10^8 cfu/mL) was added separately into a series of tubes containing 2 ml of sterile PBS at pH 2.0, 3.0 and 4.0. pH of the PBS was adjusted using a 10M solution of HCl . After 3 h of incubation, 0.1 ml from each tube was extracted and cultured on MRS agar plates. Lastly, viable colonies were counted (Conway *et al.*, 1987).

2.2.3. Proteolysis

The proteolytic activity of strains was evaluated on skim milk agar. 50 µl of cell-free supernatant was placed in the center of the plate, in a well 0.5 cm wide. Plates were then incubated at 37°C for 24 to 48 h. A clear zone around the well indicated proteolytic activity. Proteolytic activity was recorded as the diameter of the clear zone in millimeters (Nespolo, and Brandelli, 2010).

2.3.4. Degree of proteolysis (OPA assay)

Lactobacillus species were allowed to grow in milk. Following incubation, Trichloroacetic acid was added to 0.5ml of cultures to precipitate proteins. The samples were subsequently tested according to the o- phthalaldehyde (OPA) method of Church et al. (1983). The OPA solution was prepared by mixing 25 ml of 100 mM sodium tetraborate, 2.5 ml of 20% (wt/wt) SDS, 40 mg of OPA (dissolved in 1 ml of methanol), and 100 μ l of β -mercaptoethanol. To dilute the mixture, water was added to obtain the final volume of 50 ml. This reagent was freshly prepared. To evaluate proteolysis on milk proteins, a small volume (10 to 50 μ l with 5 to 100 μ g of protein) was poured directly into 1.0 ml of OPA reagent in a 1.5 ml quartz cuvette. Absorbance at 340 nm was measured with a spectrophotometer. To obtain a calibration curve absorbance was plotted against a series of serine solutions with varying concentrations ranging between 0.1 mM and 10 mM. The outcome was recorded as mmol serine l⁻¹.

2.3.5. *Lipolytic activity*

Cream fat agar plates (nutritive agar; Merck, Germany) containing 1% (v/v) milk cream (38% fat) were streaked with active cultures to qualitatively study lipolytic activity. Plates were then stored at 37°C for 72 h. Colonies surrounded by a clear ring indicated lipolytic activity. (Nieto-Arribas et al., 2009).

2.3.6. *Autolytic activity*

MRS broth cultures (Optical Density, OD₆₅₀ = 0.7–0.8) were centrifuged at 10,000 $\times$ g for 10 min at 4°C to extract exponentially growing cells. Pellets were rinsed and suspended in 20 mM sodium phosphate monobasic buffer (pH 6.8). To check for lysis, pellets were incubated for 4 hours at 30°C and changes in optical density at 650 nm were recorded using a S2000 UV/Vis spectrophotometer. Lysis was calculated using the following formula: $100 - (A_1/A_2 \times 100)$, A₁

and A₂ respectively denoting the lowest and the highest value recorded for OD₆₅₀ during the 4-hour incubation period (Lansgrud *et al.*, 1987).

2.3.7. *Exopolysaccharide and dextran production*

Bacteria were anaerobically cultured at 37°C for 72 h on of MRS agar containing 0.25% L-cysteine (MRSC agar). 2% supplementary glucose, fructose, lactose or sucrose was added to the culture separately (Ruas-Madiedo *et al.*, 2007).

A comparison was made between strains streaked onto MRS agar with 0.5 mol L⁻¹ sucrose, incubated at 30°C for 24 h, and the same strains cultured on MRS agar without added sucrose. Dextran-producing colonies could be identified using their larger size and mucous appearance when grown on the supplemented plates. *Leuconostoc mesenteroides* subsp. *mesenteroides* ATCC 8293 was used as a positive control (Herrero, *et al.* 1996).

2.3.8. *Gamma-aminobutyric acid production*

Five -mL aliquots of cultures incubated overnight at 30°C in MRS medium were centrifuged at 5,000×g, 20 min, and 25°C. The pellets were rinsed with 5 mL of a 0.9% (w/v) NaCl solution. Following a second round of centrifugation, cells were suspended in 0.5 mL of the GAD reagent solution (1 g of L-glutamic acid, 0.3 mL of Triton X-100, 90 g of NaCl, and 0.05 g of bromocresol green in 1 L of distilled water adjusted to pH 4). After 4 hours of incubation at 37°C under anaerobic conditions, plates showing no color change (yellow) were marked as negative for GAD activity. Observing a green or blue color indicated GAD activity, with blue and green revealing high and low GAD activity, respectively (Cotter *et al.*, 2001).

2.4. **Safety characteristics of *Lactobacillus* strains**

2.4.1. Biogenic amine production (BA)

The ability to form tyramine, histamine, putrescine, and cadaverine was evaluated by adding 1% (w/v) of the precursor for each amino acid; Tyrosine di-sodium salt, L-histidine monohydrochloride (Acros), L-ornithine monohydrochloride (Bio Basic, Canada) and L-lysine monohydrochloride (Acros), were added to MRS plates with 0.06% bromocresol purple (Bio Basic, Canada). The appearance of a purple color in the plates signaled positive reactions, as described by Bover Cid and Holzapfel (1999).

2.4.2. Antibiotic susceptibility

Disc Diffusion Method was used to investigate antibiotic susceptibility of strains (Fortina *et al.*, 2008). Susceptibility to four antibiotics, namely kanamycin, chloramphenicol, tetracycline (30 mg/disc) and erythromycin (15 mg/disc), was tested. Antibiotic discs were set on Mueller-Hinton agar plates inoculated with *Lactobacillus* strains that had been allowed to proliferate in MRS broth at 30°C for 24-48 h. Following incubation under the same conditions, the diameter of inhibition zones surrounding the disks was recorded. Isolates were placed in three categories based on the diameter of the inhibition zone per the Clinical and Laboratory Standards Institute tables (CLSI, 2015). The three categories included sensitive (S), intermediate (I) or resistant (R).

Statistical analysis

One-way analysis of variance (ANOVA) was performed on observations of acidifying ability, proteolytic and autolytic activity, and susceptibility to antibiotics, using the Student–Newman–Keuls (S–N–K) or Duncan test for comparison of means ($P < 0.05$) using SPSS ver.24. Duncan test was used for mean comparison.

3. Results and Discussion

3.1 . Physiological properties

After 24-h incubation in MRS broth, all strains grew well at 25, 30, 37, and 40°C. However, cultures incubated at 45°C showed stunted growth. The optimum temperature of strains like *L. brevis* (Shakibaie et al., 2017), *L. casei* (Bosnea et al., 2017) and *L. plantarum* (Hang et al., 2020) were recorded as 40°C, 45°C in yogurt and 35°C, respectively. Also, some species of lactobacillus such as *Lb. bulgaricus* show better growth at 45°C.

(Table 2). here.

3.2. Gamma-aminobutyric acid qualitative measurement

L. brevis (M2, M4 and M12) showed high GAD activity following incubation in GAD solution with pH 4.0 for 4 h at 37 °C under anaerobic conditions (Table 3). *L. casei* (M15) and *L. plantarum* isolates (M16, M17, M18 and M19) showed no GAD activity. Since, in our study, pH of GAD indicator was 4, which is the optimum pH for GABA production in *Lb. brevis* isolates that is why some of the *Lb. brevis* showed high GAD activity. (Ueno et al., 1997; Huang et al., 2007a; Huang et al., 2007b). However, in *Lb. brevis* IFO12005 the active form of GAD was proved to be a tetramer (Hiraga, et al., 2008). Our results were in agreement with those reported by Diana et al., (2014); who stated that *Lb. brevis* exhibited the highest GABA producing ability among the other LAB isolates.

Table 3. here.

LAB showing GAD activity are usually called LABGADs (Li et al., 2010). Glutamic acid decarboxylase (GAD) acts as a catalyst in the unidirectional α -decarboxylation of glutamic acid

which produces gamma-aminobutyric acid (GABA). GADs are abundant in different microorganisms including bacteria, fungi and yeasts. GADs are intracellular enzymes and their biochemical properties are known. They perform decarboxylation reactions through a similar active site, but N-terminal and C-terminal sections show great diversity. Such structural differences can influence the ability of lactobacilli to produce GABA (Shelp et al, 1999).

The food industry has indicated an interest in the production of GABA-enriched foods, as this functional compound has been endowed with hypotensive, tranquilizing, diuretic and antidiabetic effects (Hudec et al., 2017). Caseins have a high concentration of glutamic acid, thus GABA-producing LAB strains could be used as starters or adjunct cultures for the development of health-oriented dairy products (Linares et al., 2017).

3.3. Technological properties of *Lactobacillus* strains

3.3.1. Acidifying activity

After 24 h of incubation, pH changes by the *Lactobacillus* strains in this study ranged between 0.67 and 2.06 (Table 4). Thus, it seems that lactic acid production by strains was low, and only the isolates M16 ($\Delta\text{pH}=1.55$) and M19 ($\Delta\text{pH}=2.06$) of *L. plantarum*, presented moderate acidifying activity, based on the method proposed by Bouton *et al.*, (2002). In brief, sterile sugar solutions were aseptically added to broths to a final sugar concentration of 1% (w/v) in a total volume of 5 ml. Cultures were incubated at 42 C for 2 days and a positive test, corresponding to acidification, was revealed by the indicator turning yellow. One strain, *Lb. plantarum* (M19) could reduce the initial pH to less than 5 after 24 hours. This is in agreement with results obtained by Dagdemir and Ozdemir (2008), who indicated that among *Lactobacillus* isolates from artisanal Turkish White Pickled cheese, *Lb. casei* and *Lb. plantarum* strains had the highest

acidification ability and after 24 h incubation the pH of the strains was 5.32-5.66 and 5.33-5.41, respectively. Also, Ma *et al.*, (2012), showed that none of the *Lactobacillus* strains isolated from Chinese artisanal fermented milks, were characterized as fast acid producers because they did not lower the pH below 5.3 in 6 h. In fermented dairy products, the fast drop of pH, which is caused by activity of starter and adjunct cultures, leads to increase of acidity and lactose consumption. This phenomenon has impact on microbiological characteristics of fermented dairy products and shelf life of product. That's why in fermented dairy products the fast drop of pH is crucial.

Table 4. here.

Rapid acidification helps in whey drainage, inhibits the growth of most pathogens, and creates the biochemical environment required for the activity of proteolytic and lipolytic enzymes during ripening that assures the development of the cheese's typical flavor profile (Tilocca *et al.*, 2020).

It appears that all strains (except one isolate *Lb.plantarum* M19) can grow in milk with low acidification rate. This could be a desirable trait for use as probiotics or functional cultures, since after their addition, these strains do not acidify the product further. A rapid decrease in pH is very crucial for the initial step of cheese manufacture, because it is essential for coagulation and the prevention or reduction in the growth of adventitious microbiota. The *Lactobacillus* strains examined in this study, with low acidification activity (except one strain M19, *Lb. plantarum*), were unsuitable for cheese starters. However, they probably have potential use as adjunct cultures.

The ability of LAB to reduce pH through lactic acid production from sugars helps create sought-after organoleptic characteristics, inhibits establishment and growth of pathogens and guarantees the stability and safety of the final product (Ma *et al.*, 2012; Tilocca *et al.*, 2020). As a result,

production of lactic acid gives LAB a competitive edge, allowing them to dominate the culture. Lactic acid imparts the sour taste of fresh cheese and plays a role in the formation of curd.

3.3.2. Acid tolerance test

The results of investigating survival at different levels of pH revealed the acid tolerance of most strains. Generally, few bacteria survived at a pH of 2.0, some at pH 3.0 and all of them at pH 4.0. The survival of *L. brevis* M8 and M12 was very similar in pH 2.0. Almost all strains can maintain 85 percent of their initial population at pH 4.0 (Table 5).

In our study, most of lactobacilli isolates showed (0-25%) survival at pH=2 and only two isolates (M12 and M8) showed (25-50%) survival. In comparison with other results reported by Liong and shah, 2005), all isolates composed of four *Lb. acidophilus* and seven *Lb. casei*, showed tolerance to pH=2 for 2 h despite variation in degree of viability.

In our study, *L. casei* (M15) was categorized among the least tolerant strains (0-25% survival) at pH=2, whereas in those reported by Liong and shah, (2005), some of the *L. casei* strains were categorized as the most acid-tolerant strains and some of them as the most acid-sensitive strains.

Mantzourani et al., (2019), showed that 10 out of 39 lactobacillus strains displayed resistance to pH =3 and pH= 4. Among them, strains SP2 and SP5 displayed adequate viability at pH =2.

Table 5, here.

3.3.3. Proteolytic activity

The strains significantly differed with respect to proteolytic activity ($P < 0.05$). The same was true for strains from the same species (Fig.1). *L. brevis* M4 exhibited the highest proteolytic activity as indicated by detachment of amino groups. *L. brevis* M9 and *L. plantarum* M18 were

next in terms of proteolytic activity. There was no overlap between the isolates with the highest acidifying activity and those with the highest proteolytic activity, similar to other studies (Nieto-Arribas *et al*, 2009; Durlu-Ozkaya, *et al.*, 2001; Requena *et al.*, 1991).

Proteolytic activity of the starter plays a role in both acid production and flavor development. LAB break down proteins to release amino acids for their own growth and development. Despite demonstrating lower levels of proteolytic activity compared to *Bacillus*, *Pseudomonas*, or *Enterococcus*, the proteolytic activity of LAB is a major component of ripening in cheeses. (Hugenholtz, et al., 1987). Starter cultures with high proteolytic activity are used in ripening hard cheeses, as opposed to fresh unripened cheeses for which starter cultures with low proteolytic activity are favored. Starter cultures with low proteolytic activity are similarly used to ripen semi-hard cheeses for 2-3 month. Higher proteases activity compared to peptidases activity can lead to bitterness and texture defects. However, proteases are not solely to blame for the bitter taste of cheese. Other parameter of the process and enzymes used for casein coagulation (rennet, etc.) can also create a bitter taste (Coşkun, et al., 2000).

Figure 1. here

3.3.4. Degree of proteolysis

OPA spectrophotometry is a quick, easy-to-use and accurate means of quantitatively assessing proteolysis in milk and milk proteins. The OPA reagent is easy to prepare, and one solution serves for stopping hydrolysis and color development. Serine was used as a standard, since this reaction resembled the average response of other amino acids. The results of free amine groups were calculated from the standard curve. Absorption of serine solutions with 0.2, 0.4, 0.6, 0.8,

1.0 and 1.20 mg/ml was read at 340 nm and the standard curve was plotted. The results of the OPA assay for the 16 studied strains are depicted in Figure 2.

Figure 2.here

3.3.5. Lipolytic activity

Incubation on cream fat agar did not produce positive results for lipolytic activity for any of the strains. Our results were in line with those reported by Pérez et al. (2003) who worked on technological characteristics of LAB from Tenerife cheese and Nieto-Arribas et al. (2010) who worked on Manchego cheese, noting no lipolytic activity by *Lactobacillus* strains when cultured on tributyrin agar. The weak lipolytic activity seen in our samples might make the bacteria better candidates for dairy starters. Weak lipolysis of milk fat would produce aroma but not progress further to cause rancidity in the final product (Herrero *et al.*, 1996). This method has the advantage of being based on milk fat, the real substrate in cheese.

Extensive lipolysis does not occur in most cheeses. However, some hydrolysis might take place during ripening. Liberation of short chain volatile fatty acids (e.g., butyric, caproic, caprylic, and capric acid) through degradation of lipids gives Cheddar cheese its flavor. Methyl ketones, ethanol, and 2-butanol are also important flavor components in Cheddar cheese (Day et al., 1960). Several sources such as starter bacteria, added enzymes, adventitious bacteria, or milk lipase may contribute to lipolytic activity in milk. Milk lipase activity is only observed in raw milk cheeses. In hard cheeses, lipase enzymes released following the autolysis of certain *Lactobacillus* strains may contribute to lipolysis (Kayagil and Fuyagil, 2006).

3.3.6. Autolytic activity

Only certain strains are reported to possess the ability to undergo autolysis and subsequently release intercellular enzymes, so autolysis appears to be a strain-dependent property (El-Soda *et al.*, 2000). Autolysis is an advantageous trait during ripening, improving sensory and textural characteristics (Sallami *et al.*, 2004).

Fig. 3 presents the statistically significant differences in the rate of autolysis for the 16 *Lactobacillus* strains. Rates vary from 6.8% to 38.22 %. According to the classification proposed by Ayad *et al.* (2004), 25% of the strains had good autolytic properties (autolysis rates between 25% to 65%), 50% of the strains exhibited rates of autolysis between 15% and 24% and were therefore rated as fair. The remaining strains (autolysis below 14%) were categorized as poor. *L. brevis* M4 had the highest ($P < 0.05$) autolytic ability (38.22%), as well as having high proteolytic activity. Both autolytic and proteolytic activity of bacteria are thought to be necessary for quick ripening of cheese (Axelsson, 1998; Wilkinson *et al.*, 1994; Nieto-Arribas *et al.*, 2009).

Figure 3.here.

3.3.7. Extracellular polysaccharides *production and dextran production*

All 16 strains produced exopolysaccharides, most of them being mucoid (90%) and 10% being “ropy” (M5 and M8) (Figure 4). The ratio of strains that tested positive for EPS production was similar the ratio reported by Ruas-Madiedo *et al* (2007).

Figure 4 here.

None of the strains were capable of dextran production under the conditions details earlier. EPS produced by LAB has attracted increasing attention in recent years. Due to being food-grade, and GRAS (generally recognized as safe) EPS produced by LAB can be easily used in food (Badel *et*

al., 2011). An extensive body of research has revealed that some exopolysaccharides produced by LAB have remarkable biochemical and medical applications. EPS produced by LAB are reported to have immunomodulatory, antioxidant, antitumor, and cholesterol lowering activities (Kanmani et al., 2011; Luo & Fang, 2008; Tong, Xia, Feng, Sun, & Gao, 2009; Li et al., 2014).

EPS produced by LAB can form a closely attached capsule, or a loosely linked slime layer (Cerning, 1990). The formation of EPS by LAB may be harnessed to alter the physical properties of foods, through their function as viscosifying agents, stabilizers, emulsifiers, gelling agents or water-binding agents (De Vuyst et al., 2001). Other physical properties such as rheology or texture can also be modified with the addition of these molecules (Sikkema and Oba, 1998).

3.4. Safety attributes

3.4.1. Production of biogenic amines (BA)

Production of biogenic amines (BA) is an undesirable property for food-grade microorganisms (Halász, et al., 1994). Consequently, BA production by a microorganism should be studied before a recommendation for its use as a starter culture is made (Nieto-Arribas et al., 2009). Our investigation revealed that some of the 16 isolates could decarboxylate L-tyrosine, L-ornithine, L-lysine, or L-histidine (Table 6). As reported by other authors (Bover-Cid and Holzapfel, 1999; Madera et al., 2003; Roig-Sagué et al., 2002), the BA produced by the majority of LAB is tyramine, leading to its ubiquity in fermented food.

Table 6, here.

3.4.2. Susceptibility to antibiotics

The excessive use of antibiotics has spread antibiotic resistances among commensal and beneficial bacteria (Temmerman et al., 2003). There are reports of antibiotic resistance in Iranian cities due to excessive prescription of antibiotics. However, antibiotic resistance has not extensively occurred in rural areas and therefore the composition of microbial flora in rural areas remains intact. As a result, samples isolated from rural environments are prime candidates for discovery of strains that have not developed antibiotic resistance (Shanehbandi et al., 2013).

Isolates were categorized as sensitive, intermediate, or resistant (abbreviated S, I, and R respectively) based on the size of the inhibition zone after exposure to antibiotics, as proposed by the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2015). According to CLSI, all strains except M16 and M19 which were categorized as Intermediate, were resistant to kanamycin. None of the strains were resistant to erythromycin and chloramphenicol, but M16, M18 and M19 were resistant to tetracycline (Table 7).

Table 7, here.

4. Conclusions

Lactobacillus strains isolated from traditional Motal cheese showed a variety of technological characteristics. Isolates with appropriate technological characteristics such as the M4 strain, with the highest autolytic activity, *L. brevis* M4, M9 and *L. plantarum* M16 with the highest proteolytic activity, and *L. plantarum* M19 with the highest acid production, could be proposed as candidates for application as starter or adjunct cultures for cheese and other fermented foods. *L. brevis* M4 showed good proteolytic and autolytic activity and can be used as a starter or adjunct culture to produce the desired properties. Starter and adjunct cultures produce flavor molecules which contribute to ripening through the activity of proteolytic and lipolytic enzymes,

and inhibit the growth of pathogenic and spoilage microorganisms. A growing preference for natural, authentic cheeses and continued demand for consistent cheese quality with the more unique intense flavors, seems to have disappeared in industrial cheese production process. Mesophilic lactobacilli applied as adjunct cultures may provide more intense and distinctive flavor notes to cheese due to their unique metabolic attributes.

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Table 1. Identified isolates (strains) (Azizi et al., 2017).

Strain	Closest relative	Identity (%)	GenBank accession no.
M2	<i>Lactobacillus brevis</i>	%99	KX572376
M3	<i>Lactobacillus brevis</i>	%99	KX572377
M4	<i>Lactobacillus brevis</i>	%100	KX572378
M5	<i>Lactobacillus brevis</i>	%100	KX572379
M6	<i>Lactobacillus brevis</i>	%100	KX572380
M7	<i>Lactobacillus brevis</i>	%99	KX572381
M8	<i>Lactobacillus brevis</i>	%100	KX572382
M9	<i>Lactobacillus brevis</i>	%99	KX572383
M11	<i>Lactobacillus brevis</i>	%99	KX572385
M12	<i>Lactobacillus brevis</i>	%100	KX572386
M13	<i>Lactobacillus brevis</i>	%100	KX572387
M15	<i>Lactobacillus casei</i>	%100	KX572389
M16	<i>Lactobacillus plantarum</i>	%100	KX572390
M17	<i>Lactobacillus plantarum</i>	%100	KX572391
M18	<i>Lactobacillus plantarum</i>	%99	KX572392
M19	<i>Lactobacillus plantarum</i>	%100	KX572393

Table 2. Growth of the strains at different temperatures

Species	25°C	30°C	37°C	40°C	45°C
<i>L. brevis</i> (M2)	+	+	+	+	–
<i>L. brevis</i> (M3)	+	+	+	+	–
<i>L. brevis</i> (M4)	+	+	+	+	–
<i>L. brevis</i> (M5)	+	+	+	+	–
<i>L. brevis</i> (M6)	+	+	+	+	–
<i>L. brevis</i> (M7)	+	+	+	+	–
<i>L. brevis</i> (M8)	+	+	+	+	–
<i>L. brevis</i> (M9)	+	+	+	+	–
<i>L. brevis</i> (M11)	+	+	+	+	–
<i>L. brevis</i> (M12)	+	+	+	+	–
<i>L. brevis</i> (M13)	+	+	+	+	–
<i>L. casei</i> (M15)	+	+	+	+	–
<i>L. plantarum</i> (M16)	+	+	+	+	–
<i>L. plantarum</i> (M17)	+	+	+	+	–
<i>L. plantarum</i> (M18)	+	+	+	+	–
<i>L. plantarum</i> (M19)	+	+	+	+	–

Table 3. Evaluation of GABA production test using colorimetric test.

Identification based on 16S rRNA	Isolated number	GABA production evaluation
<i>L. brevis</i>	M2	+
<i>L. brevis</i>	M3	-
<i>L. brevis</i>	M4	+
<i>L. brevis</i>	M5	-
<i>L. brevis</i>	M6	-
<i>L. brevis</i>	M7	-
<i>L. brevis</i>	M8	-
<i>L. brevis</i>	M9	-
<i>L. brevis</i>	M11	-
<i>L. brevis</i>	M12	+
<i>L. brevis</i>	M13	-
<i>L. casei</i>	M15	-
<i>L. plantarum</i>	M16	-
<i>L. plantarum</i>	M17	-
<i>L. plantarum</i>	M18	-
<i>L. plantarum</i>	M19	-

Table 4. Changes of pH in Reconstituted Skim Milk (RSM) medium after 24 hours.

strains	Isolated number	ΔpH 24 h
<i>Lactobacillus brevis</i>	M2	1.09±0.07 ^d
<i>L.brevis</i>	M3	0.80±0.07 ^e
<i>L. brevis</i>	M4	0.83±0.03 ^e
<i>L. brevis</i>	M5	1.07±0.07 ^d
<i>L. brevis</i>	M6	0.69±0.04 ^f
<i>L. brevis</i>	M7	0.82±0.06 ^e
<i>L. brevis</i>	M8	1.07±0.07 ^d
<i>L. brevis</i>	M9	0.67±0.05 ^e
<i>L. brevis</i>	M11	0.80±0.03 ^e
<i>L. brevis</i>	M12	0.95±0.04 ^e
<i>L. brevis</i>	M13	1.15±0.04 ^d
<i>L. casei</i>	M15	1.13±0.04 ^d
<i>L. plantarum</i>	M16	1.55±0.06 ^b
<i>L.plantarum</i>	M17	1.46±0.05 ^b
<i>L.plantarum</i>	M18	1.3±0.05 ^c
<i>L. plantarum</i>	M19	2.06±0.06 ^a

Different superscripts in the last column means significant statistical difference (P-value<0.05).

Table 5. Survival of strains in pH 2 and pH 3 expressed as % survival.

% Survival	pH	0-25 %	25-50 %	50-75 %	75-100%
Species	2	M2, M3, M4, M5, M6, M7, M9, M11, M13, M15, M16, M17, M18, M19	M12 – M8		
	3	M3, M11, M13, M15, M17	M2, M5, M6, M16, M18, M19	M4, M7, M8	M9, M12

Table 6. Production of Biogenic amines from different precursors.

Strains	L-tyrosine	L-ornithine	L-lysine	L-histidine
<i>Lb. brevis</i> (M2)	-	-	-	+
<i>Lb. brevis</i> (M3)	-	-	+	-
<i>Lb. brevis</i> (M4)	-	-	-	-
<i>Lb. brevis</i> (M5)	-	-	+	-
<i>Lb. brevis</i> (M6)	-	-	-	-
<i>Lb. brevis</i> (M7)	+	-	-	-
<i>Lb. brevis</i> (M8)	-	+	-	-
<i>Lb. brevis</i> (M9)	-	-	-	-
<i>Lb. brevis</i> (M11)	-	-	+	-
<i>Lb. brevis</i> (M12)	-	-	-	-
<i>Lb. brevis</i> (M13)	-	-	-	-
<i>Lb. casei</i> (M15)	-	-	-	-
<i>Lb. plantarum</i> (M16)	-	-	-	-
<i>Lb. plantarum</i> (M17)	-	+	-	-
<i>Lb. plantarum</i> (M18)	-	-	-	-
<i>Lb. plantarum</i> (M19)	-	-	-	-

Table 7. The inhibition zone of routinely used antibiotics (tetracycline, erythromycin, chloramphenicol, kanamycin) against isolates.

Strains	tetracycline			erythromycin			chloramphenicol			kanamycin		
	S	I	R	S	I	R	S	I	R	S	I	R
	x≥15	12-14	x≤11	x≥23	14-22	x≤13	x≥18	13-17	x≤12	x≥18	14-17	x≤13
M2	12.33 ± 0.28(I)			22.83 ± 0.28 (S)			19.16 ± 0.28(S)			10.83 ± 0.28 (R)		
M3	15.16 ± 0.28(S)			21.83 ± 0.28 (I)			21 ± 0.5(S)			10.83 ± 0.28(R)		
M4	11 ± 0.5(I)			23.16 ± 0.28(S)			20.33 ± 0.28(S)			10.16 ± 0.28(R)		
M5	12 ± 0.5(I)			22.33 ± 0.28(S)			20.5 ± 0.5(S)			11.16 ± 0.28(R)		
M6	11.33 ± 0.28(I)			23.33 ± 0.28(S)			19.16 ± 0.28(S)			11 ± 0.5(R)		
M7	13 ± 0.5(I)			24.5 ± 0.5(S)			19.66 ± 0.28(S)			10.33 ± 0.28(R)		
M8	14.33 ± 0.28(I)			23.5 ± 0.5(S)			20.33 ± 0.28(S)			10.16 ± 0.28(R)		
M9	12.33 ± 0.28(I)			23.5 ± 0.5(S)			19.33 ± 0.28(S)			10.66 ± 0.28(R)		
M11	12.16 ± 0.28(I)			20.5 ± 0.5(I)			14.66 ± 0.28(I)			11.16 ± 0.57(R)		
M12	11.66 ± 0.28(I)			19.33 ± 0.28(I)			19.33 ± 0.28(S)			9.33 ± 0.28(R)		
M13	13.16 ± 0.28(I)			22.33 ± 0.28(S)			20.66 ± 0.28(S)			9.33 ± 0.28(R)		
M15	12.66 ± 0.28(I)			22.33 ± 0.28(S)			19.16 ± 0.28(S)			10.5 ± 0.5(R)		
M16	10.33 ± 0.28(R)			25.33 ± 0.28(S)			21.16 ± 0.28(S)			16.16 ± 0.28(I)		
M17	12.16 ± 0.28(I)			21.33 ± 0.28(I)			19.33 ± 0.28(S)			10.5 ± 0.5(R)		
M18	10.33 ± 0.28(R)			21.5 ± 0.5(I)			19.16 ± 0.28(S)			9.5 ± 0.5(R)		
M19	10.33 ± 0.28(R)			25.16 ± 0.28(S)			22.33 ± 0.28(S)			14.83 ± 0.28(I)		

R: Resistance, I: Intermediate, S: Sensitive

Figure Captions:

Figure1. Proteolytic activity of lactobacillus isolates from Motal Cheese. Mean bars without a common superscript are significantly different ($P < 0.05$) according to the Duncan test.

Figure 2. Proteolysis degree (OPA assay) of lactobacillus isolates from Motal cheese according to the Student-Newman-Keuls test.

Figure 3. Autolytic activity of *Lactobacillus* isolates from Motal Cheese. Mean bars without a common superscript are significantly different ($P < 0.05$) according to the Duncan test.

Figure 4. *Leuconostoc mesenteroides subsp.mesenteroides* ATCC 8293 as control (A), *L. brevis* M5, Dextran negative, (B); M8: EPS⁺ strain, (C).

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Credit Author Statement

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