Scientific Research Publishing

Advances in Microbiology, 2018, 8, 719-733
http://www.scirp.org/journal/aim
ISSN Online: 2165-3410
ISSN Print: 2165-3402

Characterization of *Lactobacillus* Strains Isolated from Bovine Raw Milk for Probiotic and Technological Properties

Imen Mahmoudi[1*], Olfa Ben Moussa[1], Tedj El Moulouk Khaldi[2,3], Yves Le Roux[2,3], Mnasser Hassouna[1]

[1]Unité de Recherche "Bioconservation et Valorisation des Produits Agro-Alimentaires" (UR 13AGR 02), École Supérieure des Industries Alimentaires de Tunis (ESIAT), El Khadhra, Tunis, Tunisia
[2]Université de Lorraine, Unité de Recherche "Animal et Fonctionnalités des Produits Animaux" (UR AFPA), Équipe "Protéolyse & Biofonctionnalités des Protéines & des Peptides" (PB2P), Vandoeuvre-lès-Nancy, France.
[3]INRA, Unité de Recherche "Animal et Fonctionnalités des Produits Animaux" (UR AFPA), Unité Sous Contrat 340, Vandoeuvre-lès-Nancy, France.
Email: *imenmahmoudi15@yahoo.fr

How to cite this paper: Mahmoudi, I., Ben Moussa, O., El Moulouk Khaldi, T., Le Roux, Y. and Hassouna, M. (2018) Characterization of *Lactobacillus* Strains Isolated from Bovine Raw Milk for Probiotic and Technological Properties. *Advances in Microbiology*, **8**, 719-733.
https://doi.org/10.4236/aim.2018.89048

Received: March 1, 2018
Accepted: September 16, 2018
Published: September 19, 2018

Abstract

One hundred strains isolated from bovine raw milk, obtained from different farms, were subjected to different in *vitro* stress typical of gastrointestinal tract. Twelve strains were able to tolerate pepsin at pH 2, pancreatin and bile salts (0.3%). These bacteria were identified using 16S rRNA gene sequencing. Eight isolates were *Lactobacillus plantarum* and four were *Lactobacillus fermentum*. They were not able to degrade mucin and they were *γ*-haemolytic. All strains had antibacterial activity against *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella thyphimirium*. However, only six strains inhibited *Escherchia coli*. All these showed ability for autoaggregation and/or hydrophobicity properties. They were also characterized in respect to their technological properties. Important acidification and low proteolytic and lipolytic capacities were detected for all strains. In addition, they were able to produce exopolysaccharides and grow at hot and cold temperatures. These bacteria may be used further for manufacturing of functional foods and confirming their suitability as probiotic starter cultures.

Keywords

Bovine Raw Milk, *Lactobacillus plantarum*, *Lactobacillus fermentum*, Probiotic, Functional Foods

1. Introduction

The increasing consciousness of consumption hygiene has stimulated innovation

and new products in the food industry worldwide [1]. In fact, an extensive range of probiotic functional foods are on the market around the world, most of them were from the dairy industry.

Isolation and screening of probiotic bacteria from natural sources such as raw milk have always been the most powerful means to obtain genetically-stable strains used as starters in food industry. Milk is part of the human diet and is valued as a natural and traditional food. Milk is considered to be one of the most important food groups for healthy balanced diet. As milk provides a substantial amount of nutriments in relation to its energy content, it is considered as a nutritive dense food [2].

Probiotics are alive, nonpathogenic microorganisms (bacteria or yeasts), which when administered in adequate amounts, are able to reach the intestines in sufficient numbers to confer health benefits to the host [3].

Most of probiotics are lactic acid bacteria such as *Lactobacillus* and *Bifidobacterium* species which are part of human and animal intestinal flora [4].

Key properties of probiotic bacteria rely on their ability to survive the acidic conditions, persist to the digestive enzymes present in the stomach, resist to bile salts at the beginning of the small intestine and adhere to intestinal epithelial cells and/or mucus [5]. In fact, the FAO/WHO [3] recommends verifying that a probiotic strain is safe for human consumption.

Probiotics have been designed to provide a wide diversity of beneficial effects such as antihypertensive ability, prevention of cancer, antioxidative effects, facilitation of mineral absorption and lowering of blood cholesterol levels [6]. Moreover, other properties are required for probiotic bacteria, such as desirable technological and sensory characteristics. The *Lactobacillus* strains isolated from milk and dairy products are widely used as starter in variety of food products such as yoghurt, cheese, drinks and dietary supplements [7].

Keeping in view the importance of probiotics, the present research aimed to identify candidate bacteria from Tunisian bovine raw milk with probiotic properties. As matter of fact, only the strains proved to be safe and that did show tolerance to low pH, pepsin, pancreatin and bile salts were further tested for their ability to inhibit pathogens, hydrophobicity and autoaggregation characters and evaluating their technological properties such as acidification, lipolytic and proteolytic activities, exopolysaccharides production and growth at different temperatures, in view to ameliorate their exploitation in novel functional products.

2. Materials and Methods

2.1. Isolation of Bacteria

Several samples of bovine milk were collected from dairy farms in different regions of Tunisia. One hundred presumptive lactic acid bacteria colonies were isolated five times from plates of de Man Rogosa sharpe (MRS) medium (Biokar Diagnostics) (Research Unit "Bioconservation et Valorisation des Produits

Agro-alimentaires UR 13AGR 02", ESIAT, Tunis, Tunisia). Basic characterization of the isolated strains was performed through Gram coloration, morphology and catalase tests.

2.1.1. Tolerance to the Gastrointestinal Tract

The methods used below are described according to Pennachia *et al.* [8] and Mahmoudi *et al.* [9]. Viable colony counts were enumerated after incubation at 37˚C for 3 h with pepsin, and 4 h with pancreatin, respectively.

Bile salt tolerance was determined by the method described by Anandharaj and Sivasankari [10]. Briefly, overnight cultures were inoculated in MRS broth supplemented with bile salts (oxgall, Sigma) at 0.3% (w/v). The mixture was incubated at 37˚C for 3 h and total viable count was determined following the plate method.

2.1.2. Potential Pathogenic Factors of Strains

The haemolysis test was determined by spotting on blood agar plates which was described by [11].

The mucin degradation test was determined as described by Zhou *et al.* [12] with minor modifications. Mucin (type III, Sigma, France) was supplemented to agarose medium (0.5% (w/v)) with or without glucose (3% (w/v)). 20 µL of overnight bacteria were inoculated by spotting on the medium. After incubation at 37˚C for 72 h, the plates were stained with amido black solution (0.1% (w/v) in 3.5 M acetic acid) for 30 min. Then, they were washed once with acetic acid (1.2 M). The discolored zone around the colony of *Escherchia coli* strain (DH5 alpha, Institute Pasteur of Tunisia), used as positive control, appeared. The mucin degradation activity was expressed as the size of the area of lysis of mucin.

2.1.3. Identification of Isolates

Strains, resistant to gastrointestinal conditions, were identified using 16S rRNA sequence analysis in URAFPA Research Unit and Animal Products Features—University of Lorraine—INRA—Nancy—France). 16S rRNA genes were amplified by PCR using the universal primer SSU for: TGCCAGCAGCCGCGGTA and SSU rev: GACGGGCGGTGTGTACAA. It was finished using a thermocycler as follows: denaturation at 95˚C for 3 min, 35 cycles of 30 s at 95˚C, 30 s at 55˚C, 1 min at 72˚C, 10 min at 72˚C. Amplified fragments were purified from agarose gels by a PCR purification kit (Roche). The analysis of alignment and homology of the partial nucleotide sequence of *Lactobacillus spp.* was carried out by the basic local alignment search tool (Bioedit and BLAST NCBI).

2.1.4. Antagonostic Effect

The antibacterial activity of different twelve strains against indicator pathogens such as *Salmonella thyphimurium* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* (ATCC 070 101 121) and *Escherchia coli* (DH5 alpha, Institute Pasteur of Tunisia) was tested using the agar diffusion assay which was previously described by Ben Moussa *et al.* [13].

2.1.5. Hydrophobicity Test

The degree of hydrophobicity was carried out as described by Thapa *et al.* [14]. The overnight cultures were harvested (6000 rpm, 4˚C, 5 min). After that, the cells that were once washed and resuspended in 10 mL of Ringer's solution and absorbance of aqueous phase were measured at 600 nm. Then, 10 mL of each solvent (chloroform, xylene and ethylacetate) was added to the cell suspension and the mixture was thoroughly vortexed for 2 min. After rest for 30 min, the two phases were separated and absorbance of non aqueous phase was measured at 600 nm. The adhesion of strains to organic solvents was determined by the following equation:

$$\% \text{ Hydrophobicity} = (1 - A_1/A_0) \times 100;$$

where "A_1" represents the absorbance of non aqueous phase, "A_0" represents the absorbance of aqueous phase.

2.1.6. Autoaggregation Assay

This assay was determined by Kos *et al.* [15], with some modifications. Each overnight culture was harvested (6000 rpm, 4˚C, 5 min), washed twice with PBS (pH 7.3) and resuspended in PBS to obtain OD_{595} = 0.5. 4 mL of each cell suspension was vortexed for 10 sec and incubated at 37˚C for 1 h. A_{595} of the upper layer was measured. The percentage of aggregation was expressed by the following equation:

$$\% \text{ Autoaggregation} = 1 - (A/A_0) \times 100;$$

where "A" represents the absorbance at time t = 1 h, "A_0" represents the absorbance at t = 0.

2.1.7. Technological Properties

1) *Acidification activity*

The acidifying activity of the strains was measured using titratable acidity (degree Dornic, ˚D), and pH measure according to Ben Moussa *et al.* [13]. Both parameters were determined after 6, 8, 24 and 48 h of incubation at 37˚C.

2) *Proteolytic activity*

The proteolytic activity was tested on PCA agar (Biokar Diagnostics), supplemented with 10% skim milk or 12% pork gelatine using the agar well diffusion method [16]. The proteolysis was determined by measurement of the area of halos around the inoculated spots. Moreover, this test was performed by spectrophotometric method at 345 nm, using azocasein (Sigma, France) as substrate [16].

3) *Lipolytic activity*

The production of extracellular lipases was tested on nutritive agar (Biokar Diagnostics) supplemented with 1% Tween 20, 1% Tween 80 [17]. After incubation at 37˚C for 72 h, a precipitated zone around and under the spots indicate a lipolytic activity.

Moreover, this assay was also determined by titration method as described by Ben Moussa *et al.* [13]. 1 mL of each overnight culture was inoculated in 10 mL

of MRS broth supplemented with 4% (w/v) butter fat and incubated at 37˚C for 7 days. The lipid fractions were extracted by adding 10 mL of petroleum ether (Merck) and agiting for 1 min. The free fatty acids of the upper phase (lipid extract) were titrated with NaOH (0.1 N). The lipolytic activity was determined by the following equation:

$$\% \text{ Lipolytic activity} = (a \times N \times 28.2)/g \times 100;$$

where "*a*" represents mL of NaOH used in the titration, "*N*" represents the normality of NaOH, "28.2" represents the percent equivalent weight of oleic acid and "*g*" represents the weight of fat in the sample.

2.1.8. Exopolysaccharides Production

Exopolysaccharides production (EPS) from strains was carried out as described by Van Geel-Schutten *et al.* [18]. Briefly, strains were grown in tubes containing 20 mL of MRS broth added with glucose (2% (w/v)) at 37˚C for 3 days. After centrifugation (6000 rpm, 4˚C, 20 min), two volumes of cold ethanol (Merck) (95% (v/v)) were added to one volume of culture supernatant. Precipitates were recupered by filtration under vacuum, dried at 60˚C and measured to determine the weight of EPS produced.

2.1.9. Growth at Different Non-Optimal and Cold Temperatures

According to Reale *et al.* [19], strains were tested for their ability to grow at 35, 37, 40, 42, 45 and 50˚C in MRS agar (pH 6.8) containing bromocresol purple (0.16 g/L) (Sigma, France). Plates were incubated for 48 h at different tested temperatures. After incubation, a yellow colour of substrate was considered as positive growth. For cold shock tolerance, the cultures were exposed at +4˚C and −20˚C for 24 h. After that, the cultures were incubated at 37˚C for 24 h. The OD at 595 nm was measured after the cold treatment and again after incubation [19]. Culture that is not exposed to low temperatures was considered as a control.

3. Statistical Analysis

All results were treated by one-way analysis of variance ANOVA followed by Student's test. A P value < 0.05 was considered statistically significant. Results were shown as the mean ± standard deviation of three independent experiments.

4. Results

4.1. Characterization of Strains as Potential Probiotics

4.1.1. Tolerance to the Gastrointestinal Tract and Safety Characters

In screening study, 88 strains weren't resistant to pepsin at pH 2 and only twelve out of 100 strains were resistant to pepsin at pH 2 with survival rate > 98% (Table 1). Most of the twelve strains showed a decrease in viable counts lower than 0.1 log CFU/mL. No significant differences were observed in the viability of the strains (P > 0.05). On another hand, no hymolytic and no mucin degradation activities were detected for all tested strains.

Table 1. Resistance of twelve lactic acid bacteria strains to different stress condition.

Strains	Gastrointestinal conditions		
	Pepsin[a]	Pancreatin[b]	Bile salt[c]
B24	−0.02 ± 0.13	−0.01 ± 0.01	−2.34 ± 0.5
B31	−0.02 ± 0.07	−0.02 ± 0.01	−2.51 ± 0.41
B53	−0.06 ± 0.4	−0.01 ± 0.01	−2.96 ± 0.25
B62	−0.01 ± 0.21	−0.01 ± 0.01	−2.8 ± 0.31
B66	−0.02 ± 0.14	−0.02 ± 0.01	−2.14 ± 0.37
B69	−0.02 ± 0.17	−0.01 ± 0.01	−3.49 ± 0.29
B72	−0.1 ± 0.28	−0.01 ± 0.01	−1.92 ± 0.25
B77	−0.03 ± 0.09	−0.02 ± 0.01	−2.89 ± 0.51
B81	−0.05 ± 0.02	−0.02 ± 0.01	−2.16 ± 0.18
B88	−0.04 ± 0.1	−0.02 ± 0.01	−2..3 ± 0.19
B90	−0.01 ± 0.18	−0.02 ± 0.01	−1.44 ± 0.22
B91	−0.04 ± 0.11	−0.02 ± 0.01	−3 ± 0.39

[a]Decrease of the counts (log CFU/mL) after 3 h of incubation in pepsin solution at pH 2; [b]Decrease of the counts (log CFU/mL) after 4 h of incubation in pancreatin fluid at pH 8; [c]Decrease of the counts (log CFU/mL) after 3 h of incubation with 0.3% oxgall.

4.1.2. Species Identification

16S rRNA genes sequences were determined. Eight strains were identified as *Lactobacillus plantarum* and four strains *were Lactobacillus fermentum* (Table 2). The isolated strains were identified with 100% of homology.

4.1.3. Antibacterial Activity

Table 2 shows the results of antibacterial activity of twelve strains against four pathogens. All these strains inhibited the growth of *S. aureus*, *L. monocytogenes* and *S. typhimurium*. However, only six strains inhibited the growth of *E. coli*. The highest zones of inhibition (11 ± 0.41 mm and 10 ± 0.36 mm) against *S. aureus* were observed with *L. plantarum* B72 and *L. fermentum* B90 respectively.

4.1.4. Surfaces Properties

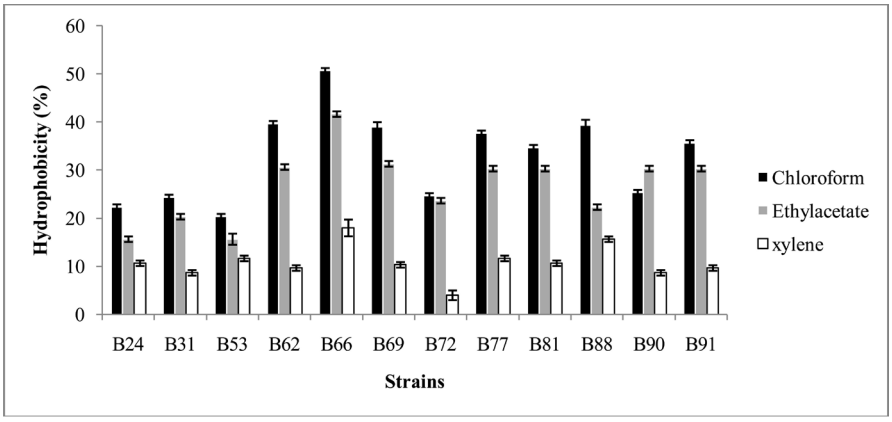
The hydrophobicity percentages of strains, using chloroform, xylene and ethylacetate, as organic solvents, are illustrated in Figure 1(a). The high values, obtained in presence of chloroform (a monopolar and acidic solvent), were 50.66% ± 0.57% for the strain B66. In addition, the strains B62, B66, B69, B77, B81, B88 and B91 have the higher levels to adhere to ethylacetate (a monopolar and basic solvent) ranged from 30% ± 0.4 to 41.66% ± 0.55% and B66 and B88 to xylene (an apolar solvent) with 18% ± 1.7% and 15.66% ± 0.6%. Our strains showed remarkable hydrophobicity.

The results of autoaggregation of twelve bacteria are depicted in Figure 1(b). The high levels obtained were 68.33% ± 1.15% and 55.34% ± 0.57% for strains B72 and B90 respectively. The strain B72 had the best autoaggregation yet its

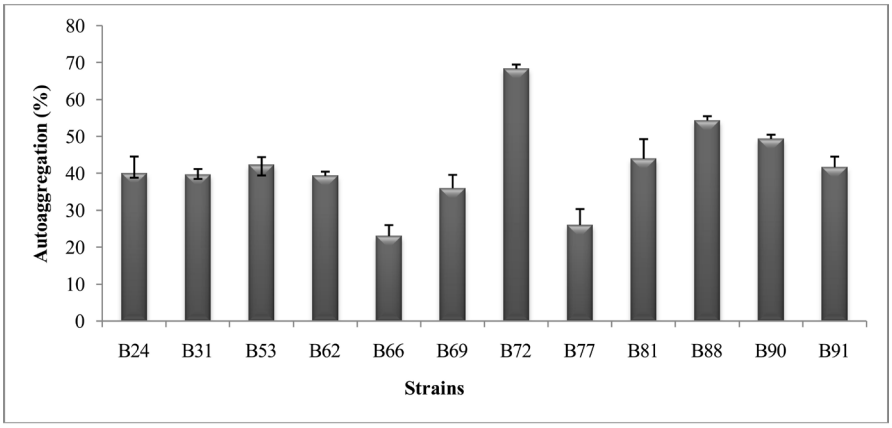
Table 2. Antimicrobial activity of twelve *Lactobacillus* strains against pathogenic bacteria.

Strains	Species	Indicator strains			
		Listeria monocytogenes	*Salmonella thyphimurium*	*Staphylococcus aureus*	*Escherihia coli*
B24	*Lactobacillus plantarum*	+	+	++	-
B31		+	+	+	-
B53		+	+	++	-
B62		+	+	++	+
B66		+	+	++	+
B69		+	+	++	+
B72		+++	++	+++	+
B77		+	+	+	-
B81		+	+	++	-
B88	*Lactobacillus fermentum*	+	+	++	+
B90		++	++	+++	+
B91		+	+	++	-

+: presence of a clear zone of growth inhibition around spots ≤ 2 mm, ++: presence of a clearly defined inhibition zone between 2 and 8 mm, +++: presence of a clearly defined inhibition zone between 8 and 12 mm and – = no inhibition.



(a)



(b)

Figure 1. Percentage of hydrophobicity (a) and autoaggregation (b) of twelve *Lactobacillus* strains.

hydrophobicity is moderate. The B66 exhibited good hydrophobicity but moderate autoaggregation.

4.2. Technological Properties

The *Lactobacillus* strains showed the highest acidifying capacities, developing to 35.33 ± 0.06˚D after 6 h and 45.33 ± 0.13˚D after 8 h (Table 3). After 48 h, the pH of inoculated milks with strains B53 and B69 decreased to values lower than 4.6 (iso-electric pH of casein) (4.15 ± 0.1 and 4.22 ± 0.2) respectively.

As concerns proteolytic activity, we observed clear zones surrounding spots on medium supplemented with skim milk for all tested strains (Table 5). *L. plantarum* B72 and *L. fermentum* B90 have largest halos, respectively 3.44 ± 0.56 mm and 3.56 ± 0.014 mm. Proteolytic activity using azocasein as substrate revealed proteloytic capacity for all selected probiotic strains. The highest OD_{345} (0.282 ± 0.33) was obtained by *L. plantarum* B77. None of the strains could've had lipolytic activity on different mediums. Otherwise, lipolytic activity, expressed by percentage of oleic acid, was very weak for all strains (Table 5). Highest value was obtained by B90 2.4% ± 0.03% fatty acids from butter fat. Furthermore, value obtained by B72 was 2.1% ± 0.01%. The B77 was the lowest lipolytic strain (0.41% ± 0.01%).

For the production of the EPS by probiotic strains, we found a concentrations ranged from 23.17 ± 0.05 mg/L for the strain B53 to 36.33 ± 0.03 mg/L for the strain B72 (Table 4).

The maximum temperatures (T_{max}) of growth of strains are shown in Table 5.

Table 3. Acidifying activity of twelve *Lactobacillus* strains.

Strains	6 h		8 h		24 h		48 h	
	pH	Acidity (˚D)	pH	Acidity (˚D)	pH	Acidity (˚D)	pH	Acidity (˚D)
Lactobacillus plantarum								
B24	5.81 ± 0.01	35.33 ± 0.06	5.62 ± 0.2	45.33 ± 0.13	5.09 ± 0.21	76.67 ± 0.05	4.26 ± 0.03	110.33 ± 0.01
B31	6.02 ± 0.02	32.33 ± 0.05	5.93 ± 0.02	43.67 ± 0.02	5.03 ± 0.07	77.33 ± 0.03	4.26 ± 0.1	104.33 ± 0.01
B53	6.05 ± 0.01	33.67 ± 0.02	5.90 ± 0.01	44.33 ± 0.02	5.20 ± 0.11	83.33 ± 0.03	4.15 ± 0.1	116.00 ± 0.01
B62	6.19 ± 0.01	30.67 ± 0.01	6.09 ± 0.22	35.33 ± 0.01	5.34 ± 0.1	75.33 ± 0.1	4.24 ± 0.1	108.33 ± 0.01
B66	6.13 ± 0.3	28.33 ± 0.01	5.97 ± 0.01	38.67 ± 0.01	5.38 ± 0.1	71.67 ± 0.1	4.22 ± 0.01	99.67 ± 0.01
B69	6.03 ± 0.01	32.67 ± 0.01	5.98 ± 0.01	43.33 ± 0.01	5.09 ± 0.09	72.33 ± 0.11	4.12 ± 0.2	113.00 ± 0.01
B72	6.08 ± 0.15	30.33 ± 0.01	6.02 ± 0.04	35.33 ± 0.08	5.21 ± 0.04	70.33 ± 0.02	4.52 ± 0.03	97.00 ± 0.01
B77	6.08 ± 0.02	30.67 ± 0.04	5.96 ± 0.01	38.33 ± 0.01	5.00 ± 0.12	82.00 ± 0.02	4.34 ± 0.04	100.33 ± 0.01
Lactobacillus fermentum								
B81	6.06 ± 0.01	30.67 ± 0.01	5.98 ± 0.01	38.67 ± 0.02	5.52 ± 0.05	67.33 ± 0.01	4.3 ± 0.02	109.67 ± 0.01
B88	6.02 ± 0.01	33.33 ± 0.01	5.98 ± 0.17	37.33 ± 0.16	5.37 ± 0.01	69.67 ± 0.05	4.45 ± 0.02	106.33 ± 0.01
B90	6.09 ± 0.01	30.33 ± 0.01	5.98 ± 0.01	36.67 ± 0.09	5.50 ± 0.2	67.33 ± 0.02	4.46 ± 0.02	95.67 ± 0.01
B91	6.08 ± 0.01	31.67 ± 0.01	5.97 ± 0.01	35.33 ± 0.01	5.36 ± 0.18	69.33 ± 0.01	4.49 ± 0.07	96.67 ± 0.01

Table 4. Proteolytic and lipolytic activities and exopolysaccharides production of twelve *Lactobacillus* strains.

Strains	Proteolytic activity		Lipolytic activity		EPS production(mg/L)
	Agar plate method (milk agar)	Azocazein method[a]	Agar plate method	Titration method (% oleic acid)	
Lactobacillus plantarum					
B24	3.24 ± 0.17	0.04 ± 0.2	-	1.77 ± 0.05	30.07 ± 0.02
B31	2.25 ± 0.29	0.013 ± 0.35	-	1.67 ± 0.01	23.50 ± 0.11
B53	3.04 ± 0.52	0.01 ± 0.14	-	1.40 ± 0.01	23.17 ± 0.05
B62	3.31 ± 0.26	0. 2 ± 0.27	-	1.77 ± 0.02	34.2 ± 0.01
B66	3.34 ± 0.31	0.215 ± 2.6	-	1.67 ± 0.1	30.33 ± 0.22
B69	3.24 ± 0.28	0.194 ± 1.45	-	1.8 ± 0.01	32.33 ± 0.12
B72	3.44 ± 0.56	0.282 ± 0.33	-	2.1 ± 0.01	36.33 ± 0.03
B77	1.69 ± 0.34	0.004 ± 0.17	-	0.41 ± 0.01	28.16 ± 0.01
Lactobacillus fermentum					
B81	3.24 ± 0.19	0.009 ± 0.6	-	1.29 ± 0.03	31.00 ± 0.05
B88	2.25 ± 0.25	0.012 ± 0.08	-	1.04 ± 0.02	28.33 ± 0.26
B90	3.36 ± 0.014	0.219 ± 0.29	-	2.40 ± 0.03	34.33 ± 0.35
B91	2.56 ± 0.3	0.06 ± 0.24	-	1.67 ± 0.01	23.33 ± 0.17

[a]: proteolytic activity according to azocasein absorbance at 345 nm. -: negative reaction.

Table 5. Maximum temperatures and growth capacities at cold shock of twelve *Lactobacillus* strains.

Strains	Maximum temperature of growth	Growth at cold shock (OD_{595} at 24 h)		
		Control (37˚C)	+4˚C	−20˚C
Lactobacillus plantarum				
B24	42 ± 0.02	0.77 ± 0.05	0.68 ± 0.2	0.66 ± 0.45
B31	42 ± 0.01	0.75 ± 0.06	0.70 ± 0.31	0.61 ± 0.29
B53	42 ± 0.01	0.82 ± 0.01	0.72 ± 0.25	0.59 ± 0.36
B62	42 ± 0.06	0.86 ± 0.03	0.80 ± 0.36	0.73 ± 0.5
B66	42 ± 0.01	0.83 ± 0.12	0.80 ± 0.55	0.68 ± 0.39
B69	45 ± 0.01	0.88 ± 0.03	0.81 ± 0.24	0.69 ± 0.14
B72	45 ± 0.15	0.89 ± 0.01	0.84 ± 0.16	0.82 ± 0.31
B77	45 ± 0.01	0.83 ± 0.09	0.76 ± 0.27	0.70 ± 0.22
Lactobacillus fermentum				
B81	45 ± 0.01	0.82 ± 0.11	0.72 ± 0.19	0.69 ± 0.51
B88	45 ± 0.01	0.80 ± 0.08	0.74 ± 0.16	0.71 ± 0.27
B90	45 ± 0.01	0.89 ± 0.01	0.85 ± 0.22	0.80 ± 0.19
B91	42 ± 0.01	0.77 ± 0.01	0.72 ± 0.18	0.61 ± 0.33

None of these was able to grow at 50˚C. In detail, the strains B24, B31, B53, B62 and B66 and B91 grew at 42˚C. While, the B69, B72, B77, B81 and B90 grew at 45˚C.

The ability to grow at 37˚C after exposure to +4˚C and −20˚C for 24 h was studied. **Table 5** shows that the strains B72 and B90 were less affected by the cold stress compared to the other tested bacteria. In fact, we reported that refrigeration and freezing have different effects on the viability of the twelve strains.

5. Discussion

Before a probiotic can present a benificial effect, it must survive during gastrointestinal passage. Most of the twelve strains showed a decrease in viable counts lower than 0.1 log CFU/mL. No significant differences were observed in the viability of the strains ($P > 0.05$). According to previous work of Solieri *et al.* [20] who reported that the critical limit survival of probiotic strains in acidic conditions was pH 2 which was efficient to inhibit the survival strains. Strains which had high survival rate were selected for good crossing of the human intestinal barrier Solieri *et al.* [20]. Besides, the membrane of the twelve strains was not affected by the enzymatic action of pepsin. Regarding the tolerance to pancreatin, similar levels of resistance were reported for all strains after 4 h of exposure, decreasing their viability less than 0.1 log CFU/mL (**Table 1**). These results were in agreement with Monteagudo-Mera *et al.* [21] who observed that the viability of strains had not been significant even when affected by this digestive protein. In fact, probiotic bacteria need to survive in the small intestine for exerting a positive effect on the human health [22]. Moreover, bile salt resistance is one of the leading conditions for any strain to be used as a probiotic. The twelve selected strains were to bile salts (oxgall) at 0.3% which has been recommended as a limit criterion for probiotic selection, after incubation for 3 h with survival rate ≥ 60%. The both strains B72 and B90 grew better than the rest of strains with a decrease in viable counts lower than 2 log CFU/mL. Anandharaj and Sivasankari [10] and Mahmoudi *et al.* [9] found that the tested strains isolated respectively from mother and camel milks respectively were resistant up to 0.3% after 3 h of incubation.

The virulence of microorganisms could be studied to ensure safety, even among a genus of bacteria that is Generally Recognised as Safe (GRAS) [3]. The twelve strains are safe and could be used as probiotics. Being identified by PCR, these species are recognized as probiotic bacteria [11] [23] and have already been reported in dairy products which are considered to be a main source of probiotics [24].

A strain could be considered as probiotic as long as it provides beneficial effects on human health, such as inhibiting pathogens that can be present in the intestine. Our strains showed important antibacterial powers. It was known that Gram positive bacteria are more sensitive to the action of lactobacilli because

they have thinner membrane than those of Gram-negative bacteria which contain many peptidoglucanes coat that may protect the cytoplasmic membrane from the antibacterial action [25]. Furthermore, Gram negative bacteria might be inhibited by lactobacilli bacteria [26].

The hydrophobicity is considered an important character which explain the behaviour of probiotic bacteria by adhesion to different substrates. Thus, all the strains are able to establish links with molecules (receptors) of the internal intestinal surface and electrostatic and/or hydrogen bonds [27]. Moreover, adherent strains can prevent the colonization of pathogens by steric interactions or specific blockage on cell receptors and provide a competitive advantage in the gastrointestinal system [28]. Rijnaarts *et al.* [29] revealed that when the hydrophobicity increases the percentage of adhesion also increases. On another side, autoaggregation and hydrophobicity are two parameters directly correlated and hydrophobicity may be a determinant of autoaggregation [30].

The most important technological characteristic to study was the acidification activity. The twelve probiotic strains are able to acidify the milk. In fact, the acid production following the pH decrease presents a specific aroma and expands the susceptibility of some organisms such as foodborne pathogens [31]. Both proteolysis and lipolysis can influence the texture and the flavor development of fermented products by formation of low molecular compounds such as free fatty acids, peptides... However, these strains don't have high proteolytic capacities compared to other genus of bacteria. But, it was revealed that the *Lactobacillus* bacteria are more proteolytic than those *Lactococcus*. Generally, lactic acid bacteria are not considered as strongly proteolytic; their proteolytic system is essential in boosting flavor development in dairy products [32]. Proteolysis could also contribute to preventing allergies frequent in children under 3 years of age due to poor digestion of milk proteins [33]. Furthermore, these probiotic strains showed negligible lipolytic activities. Again, they have an acceptable texturing character. Indeed, the amount of EPS produced by strains is strongly influenced by culture conditions such as acidity, temperature and medium composition [34]. This EPS have been revealed to have an immunomodulatory effect on intestinal epithelial cells and functions as naturally synthesised texturing agents in fermented dairy products [35]. The twelve probiotic bacteria showed thermophilic character by growth even at 45˚C. These T_{max} values were under those of Reale *et al.* [19] who reported that four *L. casei* strains grew at 48 and 49˚C, while, *L. paracasei* strains showed T_{max} values not excessing 45˚C. On the other hand, Minervini *et al.* [36] demonstrated that *L. paracasei* and *L. rhamnosus* presented a similar level of tolerance to 55˚C for 10 min that was higher than that of other mesophilic strains tested. Besides, we reported that refrigeration and freezing have different effects on the viability of the twelve strains. Compared to the control, refrigeration was less stressful than freezing after 24 h. Our results were in disagreement with Reale *et al.* [19]. Thus, refrigeration is the most frequent mean to preserve food products containing probiotic bacteria,

strategies should be conceived to improve adaptation by ameliorating the expression of protective cold protein.

Finally, this current study reveals that the twelve lactic acid bacteria isolated from bovine milk have a high probiotic potential and hence attaining all the prerequisites for probiotic use such as resistance to gastrointestinal tract, absence of undesirable properties, antimicrobial activity, hydrophobicity and autoaggregation abilities. These strains relied on technological properties such as acidification capacity, production of EPS and growth at different temperatures. Therefore, they could be used as adjunct cultures for assuring the quality and health related to functional properties of dairy products.

Acknowledgements

A special thank to Pr. Mnasser Hassouna: École Supérieure des Industries Alimentaires de Tunis (ESIAT), Tunis, Tunisia and Pr. Yves Le Roux: Université de Lorraine, Nancy, France for the realization of this work.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Vinderola, G. (2008) Dried Cell-Free Fraction of Fermented Milks: New Functional Additives for the Food Industry. *Trends in Food Science & Technology,* **19**, 40-46. https://doi.org/10.1016/j.tifs.2007.07.003

[2] Drewnowski, A. (2010) The Nutrient Rich 348 Foods Index Helps to Identify Healthy Affordable Foods. *American Journal of Clinical Nutrition*, **91**, 1095S-1101S. https://doi.org/10.3945/ajcn.2010.28450D

[3] FAO/WHO (2002) Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food. FAO/WHO, Geneva.

[4] Klein, G., Pack, G., Bonaparte, A. and Reuter, G. (1998) Taxonomy and Physiology of Probiotic Lactic Acid Bacteria. *International J ournal of Food Microbiology,* **41**, 103-125. https://doi.org/10.1016/S0168-1605(98)00049-X

[5] Sanders, M., Akkermans, L., Haller, D., Hammerman, C., Heimbach, J., Hörmannsperger, G. and Huys, G. (2010) Safety Assessment of Probiotics for Human Use. *Gut Microbiology*, **1**, 164-185. https://doi.org/10.4161/gmic.1.3.12127

[6] Ooi, L.G. and Liong, M.T. (2010) Cholesterol-Lowering Effects of Probiotics and Prebiotics: A Review of *in Vivo* and *in Vitro* Findings. *International Journal of Molecular Sciences,* **11**, 2499-2522. https://doi.org/10.3390/ijms11062499

[7] Bernardeau, M., Vernoux, J.P., Henri-Dubernet, S. and Guéguen, M. (2008) Safety Assessment of Dairy Microorganisms: The *Lactobacillus* Genus. *International Journal of Food Microbiology*, **3**, 278-285. https://doi.org/10.1016/j.ijfoodmicro.2007.08.015

[8] Pennacchia, C., Ercolini, D., Blaiatta, G., Pepe, O., Mauriello, G. and Villani, F. (2004) Selection of *Lactobacillus* Strains from Fermented Sausages for Their Potential Use as Probiotics. *Meat Science*, **7**, 309-317.

https://doi.org/10.1016/j.meatsci.2003.11.003

[9] Mahmoudi, I., Ben Moussa, O., Khaldi, T.E., Kebouchi, M., Soligot, C., Le Roux, Y. and Hassouna, M. (2016) Functional *in Vitro* Screening of *Lactobacillus* Strains Isolated from Tunisian Camel Raw Milk toward Their Selection as Probiotic. *Small Ruminant Research*, **137**, 91-98. https://doi.org/10.1016/j.smallrumres.2016.03.016

[10] Anandharaj, M. and Sivasankari, B. (2014) Isolation of Potential Probiotic *Lactobacillus oris* HMI68 from Mother's Milk with Cholesterol-Reducing Property. *Journal of Bioscience and Bioengineering*, **2**, 153-159. https://doi.org/10.1016/j.jbiosc.2014.01.015

[11] Meira, S.M.M., Helfer, V.E., Velho, R.V., Lopes, F.C. and Brandelli, A. (2012) Probiotic Potential of *Lactobacillus* spp. Isolated from Brazilian Regional Ovine Cheese. *Journal of Dairy Research*, **79**, 119-127. https://doi.org/10.1017/S0022029911000884

[12] Zhou, J.S., Gopal, P.K. and Hill, H.S. (2001) Potential Probiotic Lactic Acid Bacteria *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019) Do Not Degrade Gastric Mucin *in Vitro*. *International Journal of Food Microbiology*, **63**, 81-90. https://doi.org/10.1016/S0168-1605(00)00398-6

[13] Ben Moussa, O., Mankai, M., Setti, K., Boulares, M., Maher, M. and Hassouna, M. (2008) Characterisation and Technological Properties of Psychotropic Lactic Acid Bacteria Strains Isolated from Tunisian Raw Milk. *Annals of Microbiology*, **58**, 461-469. https://doi.org/10.1007/BF03175544

[14] Thapa, N., Pal, J., Pal, J. and Tamang, J.P. (2004) Microbial Diversity in Ngari, Hentak and Tungtap, Fermented Fish Products of North-East India. *World Journal of Microbiology and Biotechnology*, **20**, 599-607. https://doi.org/10.1023/B:WIBI.0000043171.91027.7e

[15] Kos, B., Suskovic, J., Vokavic, S., Simpraga, M., Frece, J. and Matosic, S. (2003) Adhesion and Autoaggregation Ability of Probiotic Strain *Lactobacillus acidophilus* M92. *Journal of Applied Microbiology*, **94**, 981-987. https://doi.org/10.1046/j.1365-2672.2003.01915.x

[16] Thapa, N., Pal, J. and Tamang, J.P. (2006) Phenotypic Identification and Technological Properties of Lactic Acid Bacteria Isolated from Traditionnally Processed Fish Products of the Eastern Himalayas. *International Journal of Food Microbioliogy*, **107**, 33-38. https://doi.org/10.1016/j.ijfoodmicro.2005.08.009

[17] Frank, J.F. (1997) Milk and Dairy Products. In: Doyle, M.P., Beuchat, L.R. and Montville, T.J., Eds., *Food Microbiology*, *Fundamentals and Frontiers*, American Society of Microbiology, Washington DC, 141-155.

[18] Van Geel-Schutten, G.H., Flesch, F., Brink, B., Smith, M.R. and Dijkhuizen, L. (1998) Screening and Characterization of *Lactobacillus* Strains Producing Large amounts of Exopolysaccharides. *Applied Microbiology and Biotechnology*, **50**, 697-703. https://doi.org/10.1007/s002530051353

[19] Reale, A., Di Renzo, T., Rossi, F., Zotta, T., Iacumin, L., Preziuso, M., Parente, E., Sorrentino, E. and Coppola, R. (2015) Tolerance of *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus* Strains to Stress Factors Encountered in Food Processing and in the Gastro-Intestinal Tract. *LWT Food Science and Technology*, **60**, 721-728. https://doi.org/10.1016/j.lwt.2014.10.022

[20] Solieri, L., Bianchi, A., Mottolese, G., Lemmetti, F. and Giudici, P. (2014) Tailoring the Probiotic Potential of Non-Starter *Lactobacillus* Strains from Ripened Parmigiano Reggiano Cheese by *in Vitro* Screening and Principal Component

Analysis. *Food Microbiology*, **38**, 240-249. https://doi.org/10.1016/j.fm.2013.10.003

[21] Monteagudo-Mera, A., Rodriguez-Aparicio, L., Rua, J., Martinez-Blanco, H., Navasa Nicolas, N., Garcia-Armesto, M.R. and Ferrero, M.A. (2012) *In Vitro* Evaluation of Physiological Probiotic Properties of Different Lactic Acid Bacteria Strains of Dairy and Human Origin. *Journal of Functional Foods*, **4**, 531-541. https://doi.org/10.1016/j.jff.2012.02.014

[22] Dias, F.S., Duarte, W.F., Santos, M.R.R.M., Ramos, E.M. and Schwan, R.F. (2013) Screening of *Lactobacillus* Isolated from Pork Sausages for Potential Probiotic Use and Evaluation of the Microbiological Safety of Fermented Products. *Journal of Food Protection*, **76**, 991-998. https://doi.org/10.4315/0362-028X.JFP-12-491

[23] Ramos, C.L., Thorsen, L., Schwan, R.F. and Jespersen, L. (2013) Strain-Specific Probiotics Properties of *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Lactobacillus brevis* Isolates from Brazilian Food Products. *Food Microbiology*, **36**, 22-29. https://doi.org/10.1016/j.fm.2013.03.010

[24] Xanthopoulos, V., Ipsilandis, C.G. and Tzanetakisa, N. (2012) Use of a Selected Multi-Strain Potential Probiotic Culture for the Manufacture of Set-Type Yogurt from Caprine Milk. *Small Ruminant Research*, **106**, 145-153. https://doi.org/10.1016/j.smallrumres.2012.05.005

[25] Ammor, S., Tauveron, G., Dufour, E. and Chevallier, I. (2006) Antibacterial Activity of Lactic Acid Bacteria against Spoilage and Pathogenic Bacteria Isolated from the Same Meat Smallscale Facility 1-Screening and Characterization of the Antibacterial Compounds. *Food Control*, **17**, 454-461. https://doi.org/10.1016/j.foodcont.2005.02.006

[26] Dacosta, Y. (2002) La bio-protection contre des microorganismes responsables de l'altération des aliments. In: Dacosta, Y., Ed., *La Bio-Protection des Aliments l Antagonisme Microbien au Service de la Sécurité et de la Qualité Microbiologique*, Editions Dacosta, 166-172.

[27] Bellon-Fontaine, M.N., Rault, J. and Van Oss, C.J. (1996) Microbial Adhesion to Solvents: A Novel Method to Determine the Electrondonor/Electron-Acceptor or Lewis Acid-Base Properties of Microbial Cells. *Colloids and Surfaces*, **7**, 47-53. https://doi.org/10.1016/0927-7765(96)01272-6

[28] Otero, M.C., Ocana, V.S. and Macias, E.N.M. (2004) Bacterial Surface Characteristics Applied to Selection of Probiotic Microorganisms. *Methods in Molecular Biology*, **268**, 435-440.

[29] Rijnaarts, H.H.M., Norde, W., Bouwer, E.J., Lyklema, J. and Zehnder, A.J.B. (1993) Bacterial Adhesion under Static and Dynamic Conditions. *Applied and Environmental Microbiology*, **59**, 3255-3265.

[30] Patel, A.K., Ahire, J.J., Pawar, S.P., Chaudhari, B.L. and Chincholkar, S.B. (2009) Comparative Accounts of Probiotic Characteristics of *Bacillus* spp. Isolated from Food Wastes. *Food Research International*, **42**, 505-510. https://doi.org/10.1016/j.foodres.2009.01.013

[31] Galvez, A., Abriouel, H., Ben Omar, N. and Lucas, R. (2010) Microbial Antagonists to Food-Borne Pathogens and Biocontrol. *Current Opinion in Biotechnology*, **21**, 142-148. https://doi.org/10.1016/j.copbio.2010.01.005

[32] Lopez-Kleine, L. and Monnet, V. (2011) Lactic Acid Bacteria j Proteolytic Systems. Academic Press, Cambridge. https://doi.org/10.1016/B978-0-12-374407-4.00257-0

[33] Pescuma, M., Hebert, E.M., Dalgalarrondo, M., Haertle, T., Mozzi, F., Chobert, J.-M. and Font de Valdez, G. (2009) Effect of Exopolysaccharides on the Hydrolysis of Beta Betalactoglobulin by *Lactobacillus acidophilus* CRL 636 in an *in Vitro*

Gastric/Pancreatic System. *Journal of Agricultural Food Chemistry*, **57**, 5571-5577. https://doi.org/10.1021/jf9006505

[34] Duenas-Chasco, M.T., Rodríguez-Carvajal, M.A., Tejero-Mateo, P., Espartero, J.L., Irastorza-Iribas, A. and Gil-Serrano, A.M. (1998) Structural Analysis of the Exopolysaccharides Produced by *Lactobacillus* spp. G-77. *Carbohydrate Research*, **307**, 125-133. https://doi.org/10.1016/S0008-6215(98)00034-2

[35] Patten, D.A., Leivers, S., Chadha, M.J., Maqsood, M., Humphreys, P.N., Laws, A.P. and Collett, A. (2014) The Structure and Immunomodulatory Activity on Intestinal Epithelial Cells of the EPSs Isolated from *Lactobacillus helveticus* spp. *Rosyjski* and *Lactobacillus acidophilus* spp. *Carbohydrate Research*, **384**, 119-127. https://doi.org/10.1016/j.carres.2013.12.008

[36] Minervini, F., Siragusa, S., Faccia, M., Dal Bello, F., Gobbetti, M. and De Angelis, M. (2012) Manufacture of Fior di Latte Cheese by Incorporation of Probiotic Lactobacilli. *Journal of Dairy Science*, **95**, 508-520. https://doi.org/10.3168/jds.2011-4150