Characterization of Antibacterial Activity and Potential as Probiotic of Lactic Acid Bacteria Isolated from Goat's Milk in Algeria

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Abstract: Lactic acid bacteria (LAB) were isolated from goat's milk in different north west region of Algeria. A total of 233 isolates were identified as LAB. However, only 11 strains showed excellent inhibition zones on agar when *Salmonella typhimorium* ATCC 13311 was used as an indicator for preliminary detection of antagonistic activity. After elimination of inhibition due to acid and H₂O₂, *Staphylococcus aureus* ATCC 25923 was used for secondary screening for antagonistic activity of these 11 strains. It was found that only 9 strains exhibited a good inhibition zones on agar, and all of them could inhibit *E. coli* ATCC 25921 and *Pseudomonas aeruginosa* ATCC 27853 as the third and the forth indicators respectively. *Lactococcus lactis* subsp. *cremoris*, *Lactobacillus casei* subsp. *casei*, *Lactobacillus casei* subsp. *rhamnosus*, *Streptococcus salivarius* subsp. *thermophilus* and *Lactococcus lactis* biovar. *diacetylactis* were the strains that have a good antibacterial activity, survive in the gastrointestinal conditions and were sensible to the majority of the antibiotics. All isolated strains tolerate pH = 2 and high bile salt concentration.

Key words: Goat's milk, lactic acid bacteria, antibacterial activity.

1. Introduction

In Algeria, the goat population is very high (17%), second only to sheep (28%) [1]. The caprine livestock is concentrated in the mountainous and arid areas. These goats are very adaptable to the climatic conditions and ensure the production of meat, milk and skins. Goat milk plays a vital role in human consumption, most being consumed by the rural community, while little is available on the market. Characterization and identification of the microflora from goat's milk was analyzed in different countries, like Spain [2-5], Bulgaria [6] and Algeria [7].

Lactic acid bacteria (LAB) can produce antimicrobial substances with the capacity to inhibit

the growth of pathogenic and spoilage micro-organisms. Organic acids, hydrogen peroxide, diacetyl and bacteriocins are included among these compounds [8]. Interest in naturally produced antimicrobial agents, such as bacteriocins, is on the rise, since nowadays consumers demand "natural" and "minimally processed" food [9].

The goal of probiotic therapy is to increase the number and activities of microorganisms that presumably possess health promoting properties and re-establish the normal flora [10, 11]. Commonly used bacterial probiotics include various species of *Lactobacillus*, *Bifidobacterium* and *Streptococcus*, as well as *Lactococcus lactis* and some *Enterococcus* species. Currently, the only probiotic yeast used is the nonpathogenic *Saccharomyces boulardii* [12].

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The aim of this work was, firstly, to isolate and to identify the lactic acid bacteria from Algerian goat milk. Isolates were characterized in terms of their antimicrobial activity and certain *in vitro* of characteristics related to the probiotic potential.

2. Material and Methods

2.1 Sampling

A total of 28 samples of goats' milk were collected from different regions in the north west of Algeria. Samples were immediately cooled and transported to the laboratory in an icebox (4 °C) and analyzed for content of LAB the arrival.

2.2 Isolation and Enumeration of Lactic Acid Bacteria

Ten (10) mL of milk sample were homogenized with 90 mL of sterile solution containing 0.1% (w/v) peptone (Biokar, France) and 0.85% (w/v) NaCl (Prochima, Algeria). Ten-fold serial dilutions of the meat homogenate were prepared in peptone water to spread the sample on de Man Rogosa Sharpe agar (MRS, Biokar Diagnostics, France) with 1 g/L of sorbic acid (Sigma-aldrich, USA) to inhibit yeast and molds growth. After incubation for 72 h at 30 °C, 10 colonies were randomly picked from each plate. Attention was given to choose colonies with different macroscopic morphology. Isolates were inoculated in MRS broth, incubated at 30 °C and checked for purity by streaking on MRS agar. Plates with pure cultures were used to test for cell morphology by phase contrast microscopy, Gram stain and catalase formation. Gram positive and catalase negative strains were selected. These isolates were maintained as frozen stocks in MRS broth supplemented with 10% (v/v) glycerol at -20 °C during a month. Before experimental use, all LAB strains were recovered in MRS broth and were incubated at 30 °C for 24 h.

2.3 Detection of Antagonistic Activity

The agar spot test was used for detection of antagonistic activity as described by Schillinger and

Lucke [13]. Approximately 10^4 CFU/mL of the strains to be tested for production of an antimicrobial compound were spotted onto the surface of MRS agar plates (1.5% agar) and incubated for 48 h at 37 °C to allow development of colonies. The plates were overlaid with 10 mL of nutrient soft agar (0.75% agar). The overlay agar was seeded with 10^4 CFU/mL of the pathogenic bacteria to be tested for sensitivity (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Salmonella Typhimurium*). After incubation for 24 h at 37 °C the plates were checked for inhibition zone diameters. Inhibition was scored as positive if the width of the clear zone around the colonies of the producer strain was 10 mm or larger.

Strains exhibiting antagonistic activities against pathogenic bacteria were investigated for their antimicrobial compounds as described by Aslim et al. [14]. Strains of LAB were grown for 24 h at 37 °C in 10 mL of MRS broth and then centrifuged at 9500 g for 10 min. Three samples of the supernatant were taken from each strain of LAB.

Samples 1 and 2 were adjusted to pH 6.5 with NaOH (1 N) to avoid acid inhibition. For sample 2, a catalase solution (Fluka, Germany) (300 U/mL in PBS buffer, pH = 7) was added in order to remove the inhibitory effect of hydrogen peroxide. For sample 3, the supernatant was used as a control. The antagonistic activity of the three samples was detected using the well diffusion agar method [15]. 20 mL of soft BHI agar (containing 1.0% agar) were poured on sterile plates and inoculated with an overnight culture of pathogenic bacteria (about 10^7 CFU/mL). After solidification, wells were perforated using a sterile cork borer (7.0 mm diameter). The culture filtrates (50 µL) were placed in each well. The plates were incubated at 37 °C and examined after 24 h for clear zones showing the pathogenic bacteria inhibition. The diameters of the inhibition zones were measured and the diameter of the well, 7 mm, was subtracted from the total zone diameter.

2.4 Characterization of Isolates

LAB strains were characterized according to the methods recommended by several authors [16-23]. All strains were initially subjected to Grams training, catalase test, growth at 10 °C and 45 °C in MRS and M17 broth, gas production from glucose and growth in the presence 6.5% of NaCl. Only, the strains that exhibiting antagonistic activities against pathogenic bacteria were identified by the API 50CHL identification kit (Biomerieux).

2.5 Sensitivity to Heat and Proteolytic Enzymes

Sensitivity to heat of the antibacterial compounds was investigated by treating the culture supernatants in water bath at 80 °C for 10 min and for 2 h. After heat treatment, samples were cooled to 4 °C and the residual activity was determined by the AWDA as described above. Sensitivity to proteolytic enzymes of the antibacterial compounds was investigated by the addition of Trypsin T-4799 and Proteinase K P-5056 (Sigma-Aldrich Chemie) at final concentration of 1 mg·mL⁻¹ to the culture supernatants. The samples were incubated for 3 h at 37 °C and the residual antibacterial activity was immediately determined by the AWDA as described above.

2.6 Determination of LAB Growth for Maximum Bacteriocin Production

The incubation time at which the selected LAB strain exhibited a maximum bacteriocin production was determined as follows: sterile flasks containing 50 mL of MRS broth were inoculated with 1% (v/v) of bacteriocin-producing LAB strain and incubated at 30 °C. Then, 1 mL of aliquots was retrieved after 0, 1.5, 3, 4.5, 6, 9, 12, 15, 24 and 36 h in order to monitor the production of bacteriocin along the bacterial growth. Bacteriocin production was determined by the well diffusion agar method. The diameters of inhibition zone were measured after incubation 24 h at 37 °C [24].

2.7 Survival Tests under Conditions Simulating the Human GI Tract

2.7.1 Resistance to Low pH

The methods used and described below are according to Maragkoudakis et al. [25] and Zoumpopoulou et al. [26]. Bacterial cells from overnight (18 h) cultures were harvested (10,000 × g, 5 min, 4 °C), washed twice with PBS buffer (pH = 7.2), before being re-suspended in PBS solution, adjusted to pH = 2.5. Resistance was assessed in triplicates in terms of viable colony counts and enumerated on MRS agar (BK089HA, Biokar Diagnostics) after incubation at 37 °C for 0, 0.5, 1, 2, and 3 h, reflecting the time spent by food in the stomach.

2.7.2 Resistance to Bile Salts

Bacterial cells from overnight (18 h) cultures were harvested (10,000 × g, 5 min, 4 °C) and washed twice with PBS buffer (pH7.2), before being re-suspended in PBS solution (pH8.0) containing 0.5% (w/v) bile salts (LP0055, Oxoid). Resistance was assessed in triplicates in terms of viable colony counts and enumerated after incubation at 37 °C for 0, 1, 2 and 4 h reflecting the time spent by food in the small intestine.

2.7.3 Bile Salts Hydrolysis

Fresh bacterial cultures were streaked in triplicates on MRS agar containing 0.5% (w/v) taurodeoxycholic acid (T0875, Sigma). The hydrolysis effect was indicated by different colony morphology (partial hydrolysis) from the control MRS plates, after 48 h of anaerobic incubation at 37 °C.

2.8 Statistical Analysis

Statistical analysis was performed using one-way ANOVA with post hoc Duncan's test; $P < 0.05$ was considered significant. All data are expressed as mean ± SD.

3. Results and Discussion

3.1 Isolation of Lactic Acid Bacteria

Different types of colonies developed on the surface

of MRS agar plate after 1 day of incubation at 37 °C, and after 48 or 72 h. A total of 320 strains were purely isolated and showed biochemical characteristic of lactic acid bacteria: small, round, opaque and white colonies, non-sporulated, Gram-positive, non-motile cells, catalase negative. Twenty-six strains were chosen on the basis of their antimicrobial activity relative to five reference strains.

3.2 Detection of Antagonist Activity

The antibacterial activity of 46 LAB isolates toward *Salmonella Typhimurium* was firstly determined by an agar spot test. However, only 9 strains showed excellent inhibition zones diameters larger than 40 mm, as shown in Table 1. Strain LCM07 produced the widest inhibition zone diameter on agar. The 9 LAB strains were then secondary screened for *St. aureus*, *L. monocytogenes*, *P. aeruginosa* and *E. coli* inhibition by the agar spot test. The antagonistic effects of the LAB are presented in Table 1.

Only two strains (LCS11 and LCB17) of the isolates showed low antimicrobial activity against *Listeria monocytogenes* (inhibition zones diameters < 10 mm). However, all nine isolates showed antimicrobial activity against *St. aureus* and at least one of indicator microorganisms.

It was found that antagonistic activities to ward *St. aureus* of all 9 LAB strains were lower than this capability against *S. typhimurium*.

St. aureus is Gram-positive bacteria which have a thick mesh-like cell wall made of peptidoglycan (50% to 90% of cell wall), while *S. typhimurium* is Gram-negative bacteria with a thinner layer (10% of cell wall). Gram-negative bacteria also have an additional outer membrane which contains lipids, and is separated from the cell wall by the periplasmic space. The antibacterial action of lactic acid is largely, but not totally, assigned to its ability in the undissociated form to penetrate the cytoplasmic membrane. This results in reduced intracellular pH and disruption of the transmembrane proton motive force [27]. The relative efficacy of lactic acid against Gram-negative bacteria is not unexpected. This is because the small water-soluble molecule lactic acid gains access to the periplasm through the water-filled porin proteins of the OM (outer membrane), as reviewed by Nikaido [28].

The OM, however, functions as an efficient permeability barrier that is able to exclude macromolecules (such as bacteriocins or enzymes) and hydrophobic substances (such as hydrophobic antibiotics). The permeability barrier property of the OM is largely due to the presence of a specific LPS (lipopolysaccharide) layer on the membrane surface. LPS molecules consist of a lipid part, termed lipid A, and a hydrophilic heteropolysaccharide chain protruding outward and providing the cell with a hydrophilic surface [29].

Table 1 Inhibition zone diameters of LAB isolated from goats' milk on agar when *S. typhimurium*, *St. aureus*, *P. aeruginosae* and *E. coli* were used as indicator.

Isolated No.	Inhibition zone (mm)			
	<i>S. typhimurium</i>	<i>St. aureus</i>	<i>P. aeruginosa</i>	<i>E. coli</i>
LCS01	44.2 ± 8.3*	42.3 ± 2.1	31.6 ± 5.2	ND
LCM03	41.7 ± 4.2	19.6 ± 1.8	29.7 ± 7.3	33.6 ± 6.4
LCM05	45.1 ± 5.2	31.4 ± 7.6	ND	ND
LCM07	47.3 ± 6.6	45.6 ± 2.4	25.2 ± 6.4	42.6 ± 7.8
LCS11	51.2 ± 8.1	41.5 ± 7.5	ND	24.5 ± 3.2
LCS13	42.8 ± 2.1	33.7 ± 2.7	ND	27.5 ± 5.8
LCS15	44.3 ± 5.0	21.3 ± 3.2	27.3 ± 7.7	18.3 ± 6.7
LCB17	46.6 ± 3.3	44.5 ± 4.3	18.6 ± 6.3	ND
LCB26	45.0 ± 3.9	43.1 ± 5.2	ND	41.1 ± 4.4

*Mean ± SD from triplicate determinations. ND: not determinated.

In the present study, it was found that LCM07, LCB17 and LCB26 produced the widest inhibition zones when *St. aureus* was used as an indicator. In addition, for a third screening against *E. coli* and inhibition, only 2 strains exhibited inhibition zones on agar, as shown in Table 1. The strains which showed the widest zones of inhibition against *E. coli* were LCM07 and LCB26.

The widest inhibition zone was produced by LCS01 when *P. aeruginosa* was used. Roth and Keenan [30] reported that lactic acid is able to cause sub-lethal injury to *E. coli*, and similar properties have also been assigned to acetic acid [31]. Indirect evidence suggests that such injury involves disruption of the LPS layer. A permeabilizer function of lactic acid would not only be utilizable in decontamination procedures and in protective cultures. It would also provide a mechanistic explanation for the antimicrobial and health-promoting effects of probiotic lactic acid bacteria [32].

Lactic acid, in addition to its antimicrobial property due to the lowering of the pH, also functions as a permeabilizer of the Gram negative bacterial outer membrane. It may also act as a potentiator of the effects of other antimicrobial substances [33].

Three LAB strains (LCS1, LCM05 and LCB26) which exhibited inhibitory efficiency toward tested pathogenic bacteria were analyzed for their production of antibacterial compounds. Supernatants obtained from all strains did not exhibit the inhibition zones after pH adjustment to 6.5. It indicated that the inhibitory effect toward pathogenic bacteria mainly because of organic acid. LAB produces various compounds such as organic acids, diacetyl, hydrogen peroxide, and bacteriocin or bactericidal proteins during lactic fermentations. The bacteriocins from them are generally recognized as safe (GRAS) and LAB have attracted a great deal of attention as a novel approach to control pathogens in food-stuffs [34]. Levels and types of organic acids produced during the fermentation process depend on LAB species or

strains, culture composition and growth conditions [35]. Hydrogen peroxide is produced by LAB in the presence of oxygen as a result of the action of flavoprotein oxidases or NADH (nicotinamide adenine dinucleotide) peroxidase. The antimicrobial effect of hydrogen peroxide may result from the oxidation of sulfhydryl groups causing denaturing of a number of enzymes, and from the peroxidation of membrane lipids thus increasing membrane permeability [36, 37].

The antimicrobial effect of organic acids lies in the reduction of pH, as well as the undissociated form of the molecules. It has been proposed that the low external pH causes acidification of the cell cytoplasm, while the undissociated acid, being lipophilic, can diffuse passively across the membrane. The undissociated acid acts by collapsing the electrochemical proton gradient, or by altering the cell membrane permeability which results in disruption of substrate transport systems [38].

3.3 Identification of Selected Strains

The nine strains which exhibited widest zones of inhibition were identified as *Streptococcus salivarius* subsp. *thermophilus* (LCS01), *Leuconostoc lactis* (LCM03 and LCS13), *Lactococcus lactis* subsp. *lactis* (LCM05), *Lactococcus lactis* subsp. *cremoris* (LCM07), *Lactococcus lactis* biovar. *diacetylactis* (LCS11 and LCS15), *Lactobacillus casei* subsp. *casei* (LCB17) and *Lactobacillus casei* subsp. *ramnosus* (LCB26), as shown in Table 2.

3.4 Effect of Heat and Proteolytic Treatments on Antibacterial Activity

Application of Trypsin T-4799 and Proteinase KP-5056 (Sigma-Aldrich Chemie) at final concentration of 1 mg·mL⁻¹ led to inactivation of the antagonistic activities of culture supernatants. Heating at 80 °C for 10 min did not affect the antibacterial compounds since the AWDA showed residual activities of samples. However, no inhibition zones

Table 2 Carbohydrates fermentation of lactic acid bacteria isolated from raw goats' milk.

Species	Star	Amy	Ara	Cel	Fru	Gal	Glu	Lac	Mal	Man	Mos	Mel	MLz	Raf	Rha	Rib	Suc	Sal	Sor	Tre	Xyl
LCS01	-	-	-	-	-	+	+	+	-	-	-	-	-	v	-	-	+	-	-	-	-
LCM03	-	-	-	-	+	+	+	+	+	-	v	-	v	v	-	-	-	-	-	-	-
LCM05	+	-	-	+	+	+	+	+	+	-	+	-	-	-	-	+	-	+	-	v	v
LCM07	+	-	-	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	v
LCS11	-	-	-	-	+	+	+	+	+	-	+	-	-	-	-	+	+	-	+	-	+
LCS13	-	-	-	-	+	+	+	+	+	-	v	-	v	v	-	-	-	-	-	-	-
LCS15	-	-	-	-	+	+	+	+	+	-	+	-	-	-	-	+	+	-	+	-	+
LCB17	-	+	-	+	+	+	+	-	v	+	+	+	v	-	-	-	-	+	-	+	-
LCB26	-	+	-	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	+	-

+: Positive reaction; -: Negative reaction; v: variable.

LCS01: *Streptococcus salivarius* subsp. *thermophilus*; LCM03: *Leuconostoc lactis*; LCM05: *Lactococcus lactis* subsp. *lactis*; LCM07: *Lactococcus lactis* subsp. *cremoris*; LCS11: *Lactococcus lactis* biovar. *diacetylactis*; LCS13: *Leuconostoc lactis*; LCS15: *Lactococcus lactis* biovar. *diacetylactis*; LCB17: *Lactobacillus casei* subsp. *casei*; LCB26: *Lactobacillus casei* subsp. *rhamnosus*.

Star: Starch; Amy: Amygladin; MLz: Melizitose; Sor: Sorbitol; Tre: Trealose; Ara: Arabinose; Raf: Raffinose; Xyl: Xylose; Rib: Ribose; Suc: Sucrose; Rha: Rhamnose; Cel: Cellubiose; Mel: Melibiose; Sal: Salicin; Man: Mannitol; Mos: Mannose; Fru: Fructose; Gal: Galactose; Glu: Glucose; Lac: Lactose; Mal: Maltose.

Fig. 1 Inhibition zones after heating treatment (LCS01: *Streptococcus salivarius* subsp. *thermophilus*; LCM03: *Leuconostoc lactis*; LCM05: *Lactococcus lactis* subsp. *lactis*; LCM07: *Lactococcus lactis* subsp. *cremoris*; LCS11: *Lactococcus lactis* biovar. *diacetylactis*; LCS13: *Leuconostoc lactis*; LCS15: *Lactococcus lactis* biovar. *diacetylactis*; LCB17: *Lactobacillus casei* subsp. *casei*; LCB26: *Lactobacillus casei* subsp. *rhamnosus*).

were observed after heating at 80 °C for 2 h, as shown in Fig. 1.

Antibacterial activity could be detected only on agar plated in overlay assays, and was unsuccessfully researched in cell-free culture supernatant fluids under conditions that eliminate acid and hydrogen peroxide inhibition.

Therefore regarding the proteinaceous nature, the thermostability at 80 °C for 10 min of antibacterial compounds and their residual activities after ruling out acid and hydrogen peroxide inhibition, it was

concluded that theses antibacterial compounds were bacteriocin-like [39]. It must also be kept in mind that the production and extraction of bacteriocins may be affected by a range of factors. Among these it is possible to cite the composition of the culture medium, including carbon, nitrogen and sources of phosphates, cations, surfactant and inhibiting agents, the optimum pH, aeration and growth temperature [40-43]. Generally, for the maximum production of bacteriocins it is necessary to have a complex medium whose pH does not usually coincide with the optimum

pH for bacteriocin action [41]. In addition, further factors may have an influence, such as the adsorption of bacteriocins on the cell wall of the producing micro-organisms. This effect may well be the prime mechanism causing a decline in bacteriocin activity, followed by hydrolysis of proteins [42, 44].

3.5 Determination of LAB Growth for Maximum Antibacterial Agent Production

Maximum inhibitory activity against *St. aureus* was found at 24 h of incubation at 30 °C, which corresponded to the stationary phase, as shown in Fig. 2. Likewise, maximum inhibitory activity of all strains against *St. aureus* was detected after 15 h of incubation, coinciding with the beginning of the stationary phase of growth in Fig. 2. In both cases, after time mentioned no further increase of inhibitory activity was found. Moreover, a decrease of 50% in the antimicrobial activity of the bacteriocin-producing strain LCB17 and LCB26 against *St. aureus* was observed at 36 h of incubation.

A number of factors must be taken into consideration when choosing a bacteriocinogenic strain for bacteriocin *in situ* or *ex situ* production. Among several factors, presence and concentration of food additives are master keys for an optimum performance of a bacteriocinogenic strain in a producing medium and especially for lacto bacilli strains, the decrease in pH of the medium has a significant effect on the production of bacteriocin [45]. Hence, the quantity of the response may be specific for the combinations of bacteriocins, food additives and target organisms.

3.6 Survival under Conditions Simulating the Human GI Tract

In order to act as a probiotic in the gastrointestinal tract and to exert their beneficial effect on the host, the bacteria must be able to survive the acidic conditions in the stomach and resist bile acids at the beginning of the small intestine [46, 47].

Fig. 2 LAB growth for maximum antibacterial agent production (LCS01: *Streptococcus salivarius* subsp. *thermophilus*; LCM03: *Leuconostoc lactis*; LCM05: *Lactococcus lactis* subsp. *lactis*; LCM07: *Lactococcus lactis* subsp. *cremoris*; LCS11: *Lactococcus lactis* biovar. *diacetylactis*; LCS13: *Leuconostoc lactis*; LCS15: *Lactococcus lactis* biovar. *diacetylactis*; LCB17: *Lactobacillus casei* subsp. *casei*; LCB26: *Lactobacillus casei* subsp. *rhamnosus*).

Approximately 2.5 L of gastric juice [48] and 1 L of bile [49] are secreted into the human digestive tract every day. Thus, it is essential for the bacteria to have protection systems to withstand the low pH in the stomach, digestive enzymes and bile in the small intestine.

3.6.1 Resistance Ability to Low pH

The nine strains of LAB showed higher resistance to low pH, whilst variability in the final counts was obtained. Table 1 shows the ranges of the final counts of all the tested strains after exposure to low pH for 3 h, whereas Fig. 3 shows the viable counts of the most resistant LAB strains (final counts > 8 log CFU/mL) after 0, 0.5, 1, 2, and 3 h incubation in pH 2.5. Overall, 3 strains showed very high resistance to low pH (LCB26, LCB17 and LCM07) with final populations exceeding 8 log CFU/mL, whereas 6 strains showed high resistance to low pH (6 and 8 log CFU/mL).

These results are in agreement with those obtained from previous studies, where *Lactobacillus* strains of food, human or animal origin, were able to retain their viability when exposed to pH values of 2.5 and 4.0 [25, 50-54].

Fig. 3 Resistance for low pH of the selected strains (LCS01: *Streptococcus salivarius* subsp. *thermophilus*; LCM03: *Leuconostoc lactis*; LCM05: *Lactococcus lactis* subsp. *lactis*; LCM07: *Lactococcus lactis* subsp. *cremoris*; LCS11: *Lactococcus lactis* biovar. *diacetylactis*; LCS13: *Leuconostoc lactis*; LCS15: *Lactococcus lactis* biovar. *diacetylactis*; LCB17: *Lactobacillus casei* subsp. *casei*; LCB26: *Lactobacillus casei* subsp. *rhamnosus*).

Table 3 Tolerance of LAB strains at different bile concentrations for 4 h of incubation.

Bile salt (%)	Log CFU/mL (SD) ^a								
	LCS01	LCM03	LCM05	LCM07	LCS11	LCS13	LCS15	LCB17	LCB26
0	6.50 ± 0.10	7.12 ± 0.02	7.18 ± 0.02	7.33 ± 0.06	6.90 ± 0.11	6.50 ± 0.10	7.44 ± 0.03	7.02 ± 0.07	7.50 ± 0.10
0.5	6.48 ± 0.08	7.04 ± 0.14	7.02 ± 0.02	7.12 ± 0.05	6.81 ± 0.04	6.48 ± 0.08	7.28 ± 0.07	7.01 ± 0.06	7.48 ± 0.03
1	5.89 ± 0.12	5.14 ± 0.06	7.01 ± 0.04	7.09 ± 0.16	5.98 ± 0.07	5.89 ± 0.12	7.02 ± 0.11	7.01 ± 0.12	7.29 ± 0.02
1.5	5.77 ± 0.09	3.37 ± 0.29	7.07 ± 0.06	6.87 ± 0.11	5.93 ± 0.03	3.77 ± 0.09	5.27 ± 0.12	6.79 ± 0.04	7.17 ± 0.05

^a: Bacterial levels were determined by plate counts on MRS spread plates. Data are presented as mean (standard deviations).

Several assays *in vitro* have been made to select acid resistant strains by using exposure to pH-adjusted PBS [50, 55], incubation in gastric contents [56] and use of a dynamic model of the stomach [57].

Conway et al. [43] found survival of lactobacilli to be slightly lower when PBS was used rather than gastric juice, because components in the gastric juice could have some protective effect on the bacterial cell. They suggested the use of PBS at the desired pH to screen strains for their ability to maintain viability *in vivo* when exposed to gastric juice. Moreover, the probiotic strains could be buffered by food or other carrier matrix molecules following consumption and are thus not likely to be exposed to the pH of the stomach [58].

3.6.2 Resistance to Bile Salts and Bile salts Hydrolysis

Most of the BAL isolates were able to grow in

media added with 0.5%, 1% and 1.5% bile and only 2 were sensitive to the three concentrations of bile evaluated. While the bacilli were able to resist 1.5% bile concentration, *Leuconostocs* present a sensibility for this highest concentration, as shown in Table 3.

The majority of tested strains were found to be resistant to bile salts even after 4 h of exposure retaining their viability with negligible reduction in viable counts. Only 2 LAB strains (LCM03 and LCS11) demonstrated approximately 3 log reduction after exposure to highest bile salts concentration. Regarding bile salt hydrolysis, 12 strains exhibited partial bile salt hydrolase activity, recorded as differentiated colony morphology on TDCAMRS agar in comparison with the control MRS agar plates. These were the 2 *lactobacilli* (LCB17 and LCB26) strains.

Bile plays a fundamental role in specific [57] and non-specific [59] defense mechanism of the gut and the magnitude of its inhibitory effect is determined primarily by the bile salts concentration [60]. Therefore, bile tolerance is considered as an important characteristic of *Lactobacillus* strains, which enables them to survive, grow, and exert their action in gastrointestinal transit. According to Sanders et al. [61], *Lactobacillus* strains which could grow and metabolize in normal physical bile concentration could survive in gastrointestinal transit. The resistance to bile salt of some strains was related also to the activity of bile salt hydrolase which can hydrolyze combined bile salt and thus reduce their toxic and side effects. In addition, Gänzle et al. [62] and Du Toit et al. [51] proposed that some components of food could protect and promote the resistance of strain to bile salt.

Bile salt hydrolysis has been correlated to cholesterol lowering [63, 64]. One hypothesis proposes that the cholesterol lowering property of *Lactobacillus* is due to incorporation of cholesterol into the cellular membranes of bacteria from the medium during its growth period [65]. The main mechanism however is linked to the bile salt hydrolase activity of the cells [66]. Indeed some strains of LAB secrete bile salt hydrolase enzyme, which hydrolyses conjugated bile acids to release de-conjugated bile acids and amino acids [63, 67, 68]. When these salts are secreted from the gastrointestinal tract, the demand for cholesterol is increased, which in turn lowers cholesterol levels [69, 70]. On the other hand, it is yet not completely clear whether BSH activity is a desirable property for probiotics, since large amounts of de-conjugated bile salts may have undesirable effects for the human host [71, 72]. However, current research data strongly suggest that microbial BSH function in the detoxification of bile salts increases the intestinal survival and persistence of producing strains, which in turn increases the overall beneficial effects associated with the strain [49, 63]. Moreover, the bacterial genera that would most

likely be used as probiotics (bifidobacteria and lactobacilli) are not capable of dehydroxylating de-conjugated bile salts [73-75], and thus the majority of the breakdown products of BSH activity by a probiotic strain may be precipitated and excreted in feces [76, 77].

4. Conclusion

Based on this study, it can be concluded that the Algerian goat's milk contains a variety of lactic acid bacteria display a significant antibacterial activity against pathogenic bacteria. The isolated strains show a good tolerance to gastrointestinal tract conditions and they can be exploited as a promising potential probiotics.

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References

- [1] Agricultural Statistics, 1990-2009, Series D areas and Production, National Center for Statistics, Ministry of Agriculture Algeria, 2009, pp. 22-23.
- [2] J. Fontecha, C. Pelaez, M. Juarez, T. Requena, C. Gomez, Biochemical and microbiological characteristics of artisanal hard goat's milk cheese, *Journal of Dairy Science* 73 (1990) 1150-1157.
- [3] T. Requena, M.A. De la Fuente, P. Fernandez de Palencia, M. Juarez, C. Pelaez, Evaluation of a specific starter for the production of semi-hard goat's milk cheese, *Lait* 72 (1992) 437-448.
- [4] C. Olarte, S. Sanz, E. Gonzales-Fandos, P. Torre, The effect of a commercial starter culture addition on the ripening of an artisanal goat's milk cheese (Cameros cheese), *Journal of Applied Microbiology* 88 (2000) 421-429.
- [5] I. Sanchez, S. Sesena, J.M. Poveda, L. Cabezas, L. Palop, Phenotypic and genotypic characterization of lactobacilli isolated from Spanish goat cheeses, *International Journal of Food Microbiology* 102 (2005) 355-362.
- [6] L. Tserovska, S. Stefanova, T. Yordanova, Identification of lactic acid bacteria isolated from Katyk, goat's milk and cheese, *Journal of Culture Collection* 3 (2002) 48-52.
- [7] A. Badis, D. Guetarni, B. Moussa Boudjemaa, D.E.

- Henni, M. Kihal, Identification and technological properties of lactic acid bacteria isolated from raw goat milk of four Algerian races, *Food Microbiology* 21 (2004) 579-588.
- [8] M.A. Daeschel, Antimicrobial substances from lactic acid bacteria for use as food preservatives, *Food Technology* 43 (1989) 164-166.
- [9] J. Cleveland, T.J. Montville, I.F. Nes, M.L. Chikindas, Bacteriocins: Safe, natural antimicrobials for food preservation, *International Journal of Food Microbiology* 71 (2001) 11-20.
- [10] L.J. Fooks, R. Fuller, G.R. Gibson, Prebiotics, probiotics and human gut microbiology, *International Dairy Journal* 9 (1999) 53-61.
- [11] E. Tsakalidou, Probiotic potential of *Lactobacillus* strains isolated from dairy products, *International Dairy Journal* 16 (3) (2006) 189-199.
- [12] L.E. Morrow, V. Gogineni, M.A. Malesker, Probiotics in the intensive care unit, *Nutrition Clinical Practice* 27 (2) (2012) 235-241.
- [13] U. Schillinger, F.K. Lucke, Antibacterial activity of *Lactobacillus sake* isolated from meat, *Applied and Environmental Microbiology* 55 (1989) 1901-1906.
- [14] B. Aslim, Z.N. Yuksekdog, E. Sarikaya, Y. Beyatli, Determination of the bacteriocin-like substances produced by some lactic acid bacteria isolated from Turkish dairy products, *LWT Food Science and Technology* 38 (6) (2005) 691-694.
- [15] E. Papamalonis, N. Tzanetakis, E.L. Tzanetaki, P. Kotzekidou, Characterization of lactic acid bacteria isolated from a Greek dry-fermented sausage in respect of their technological and probiotic properties, *Meat Science* 65 (2) (2003) 859-867.
- [16] W.F. Harrigan, M.E. McCance, *Laboratory Methods in Food and Dairy Microbiology*, 2nd ed., Academic Press, London, 1976, pp. 320-321.
- [17] M.E. Sharpe, Identification of the lactic acid bacteria, in: F.A. Skinner, D.W. Lovelock (Eds.), *Identification Methods for Microbiologist*, Academic Press, London, 1979, pp. 233-259.
- [18] O. Kandler, N. Weiss, Regular, non-spore-forming Gram positive rods, in: P.H.A. Sneath, N. Mair, M.E. Sharpe, J.G. Holt (Eds.), *Bergey's Manual of Systematic Bacteriology*, Williams & Wilkins, Baltimore, Vol. 2, 1986, pp. 1208-1234.
- [19] K.H. Schleifer, R. Kilpper-Balz, Molecular and chemotaxonomic approaches to the classification of streptococci, enterococci and lactococci: A review, *Systematic Applied Microbiology* 10 (1987) 1-19.
- [20] R.R. Facklam, M.D. Collins, Identification of *Enterococcus* species isolated from human infections by a conventional testscheme, *Journal of Clinical Microbiology* 27 (1989) 731-734.
- [21] M.C. Curk, J.C. Hubert, F. Bringel, *Lactobacillus paraplantarum* sp. nov., a new species related to *Lactobacillus paraplantarum*, *International Journal of Systematic Bacteriology* 46 (1996) 595-598.
- [22] W.P. Charteris, P.M. Kelly, L. Morelli, J.K. Collins, Quality control *Lactobacillus* isolates for use with the API 50CH and API ZYM systems at 37 °C, *Journal of Basic Microbiology* 41 (2001) 241-251.
- [23] G. Klein, International Committee of Systematic Bacteriology, subcommittee on the taxonomy of *Bifidobacterium*, *Lactobacillus* and related organisms, Minutes of the meeting, *International Journal of Systematic Evolution Microbiology* 51 (2001) 259-261.
- [24] S. Kastner, V. Perreten, H. Bleuler, G. Hugenschmidt, C. Lacroix, L. Meile, Antibiotic susceptibility patterns and resistance genes of starter cultures and probiotic bacteria used in food, *Systematic Applied Microbiology* 29 (2006) 145-155.
- [25] P.A. Maragkoudakis, G. Zoumpopoulou, C. Miaris, G. Kalantzopoulos, B. Pot, E. Tsakalidou, Probiotic potential of *Lactobacillus* strains isolated from dairy products, *International Dairy Journal* 16 (2006) 189-199.
- [26] G. Zoumpopoulou, B. Foligne, K. Christodoulou, C. Grangette, B. Pot, E. Tsakalidou, *Lactobacillus fermentum* ACA-DC 179 displays probiotic potential *in vitro* and protects against TNBS-induced colitis and *Salmonella* infection in murine models, *International Journal of Food Microbiology* 121 (2008) 18-26.
- [27] B. Ray, W.E. Sandine, Acetic, propionic, and lactic acids of starter culture bacteria as Biopreservatives, in: B. Ray, M. Daeschel (Eds.), *Food Biopreservatives of Microbial Origin*, Boca Raton, CRC Press, Fla, 1992, pp. 103-136.
- [28] H. Nikaido, Outer membrane, in: F.C. Neidhardt (Ed.), *Escherichia coli and Salmonella: Cellular and Molecular Biology*, American Society for Microbiology, Washington, D.C, 2nd ed., 1, 1996, pp. 29-47.
- [29] I.M. Helander, Y. Kato Kilpelainen, I. Kostinen, R. Lindner, B. Nummila, K. Sugiyama, et al., Characterization of lipopolysaccharides of polymyxin-resistant and polymyxin-sensitive *Klebsiella pneumoniae* O₃, *European Journal of Biochemistry* 237 (1996) 272-278.
- [30] L.A. Roth, D. Keenan, Acid injury of *Escherichia coli*, *Canadian Journal of Microbiology* 17 (8) (1971) 1005-1008.
- [31] K.S. Przybylski, L.D. Witter, Injury and recovery of *Escherichia coli* after sublethal acidification, *Applied and Environmental Microbiology* 37 (2) (1979) 261-265.
- [32] S. Salminen, M.A. Deighton, Y. Benno, S.L. Gorbach, Lactic acid bacteria in health and disease, in: S. Salminen, A. vonWright (Eds.), *Lactic Acid Bacteria: Microbiology*

- and Functional Aspects, Marcel Dekker, Inc., New York, 1998, pp. 211-253.
- [33] H.L. Alakomi, E. Skyttä, M. Saarela, T. Mattila-Sandholm, K. Latva-Kala, I. Helander, Lactic acid permeabilizes Gram-negative bacteria by disrupting the outer membrane, *Applied and Environmental Microbiology* 66 (5) (2000) 2001-2005.
- [34] A. Savadogo, C.A.T. Ouattara, I.H.N. Bassole, A.S. Traore, Antimicrobial activities of lactic acid bacteria strains isolated from Burkina faso fermented milk, *Pakistan Journal of Nutrition* 3 (3) (2004) 174-179.
- [35] S.E. Lindgren, W.J. Dobrogosz, Antagonistic activities of lactic acid bacteria in food and feed fermentations, *FEMS Microbiology Reviews* 7 (1990) 149-163.
- [36] S. Condon, Responses of lactic acid bacteria to oxygen, *FEMS Microbiology Letters* 46 (3) (1987) 269-280.
- [37] S. Kong, A.J. Davison, The role of interactions between O_2 , H_2 , OH , e^- and O_2^- in free radical damage to biological systems, *Archives of Biochemistry and Biophysics* 204 (1) (1980) 18-29.
- [38] S. Ammor, G. Tauveron, E. Dofour, I. Chevallier, Antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility: 1. Screening and characterization of the antibacterial compounds, *Food Control* 17 (6) (2006) 454-461.
- [39] S. Ammor, C. Rachman, S. Chaillou, H. Pre'vost, X. Dousset, M. Zagorec, et al., Phenotypic and genotypic identification of lactic acid bacteria isolated from a small-scale facility producing traditional dry sausages, *Food Microbiology* 22 (2005) 373-382.
- [40] V. Carolissen-Mckay, G. Arendse, J.W. Mastings, Purification of bacteriocins of lactic acid bacteria: Problems and pointers, *International Journal of Food Microbiology* 34 (1997) 1-16.
- [41] H. Daba, C. Lacroix, J. Huang, R.E. Simard, In Xuence of growth conditions on production and activity of mesenterocine 5 by a strain of *Leuconostoc mesenteroides*, *Applied Microbiology and Biotechnology* 39 (1993) 166-173.
- [42] E. Parente, A. Ricciardi, Production, recovery and purifications of bacteriocins from lactic acid bacteria, *Applied Microbiology and Biotechnology* 52 (1999) 628-638.
- [43] J.R. Tagg, A.S. Dajani, L.W. Wannamaker, Bacteriocins of Gram-positive bacteria, *Bacteriological Reviews* 40 (1976) 722-726.
- [44] A.K. Bhunia, M.C. Jonson, B. Ray, N. Kalchayanand, Mode of action of pediocin AcH from *Pediococcus acidilactici* sensitive bacterial strains, *Journal of Applied Bacteriology* 70 (1991) 25-30.
- [45] M. Gänzle, S. Weber, W. Hammes, Effect of ecological factors on the inhibitory spectrum and activity of bacteriocins, *International Journal of Food Microbiology* 46 (1999a) 207-217.
- [46] W.H. Holzapfel, P. Haberer, J. Snel, V. Schillinger, J. Huisin't Veld, Overview of gut flora and probiotics, *International Journal of Food Microbiology* 41 (1998) 85-101.
- [47] T.R. Klaenhammer, M.J. Kullen, Selection and design of probiotics, *International Journal of Food Microbiology* 50 (1999) 45-57.
- [48] P.D. Cotter, C. Hill, Surviving the acid test: Responses of Gram-positive bacteria to low pH, *Microbiology and Molecular Biology Reviews* 67 (2003) 429-453.
- [49] M. Begley, G.G. Gahan, C. Hill, The interaction between bacteria and bile, *FEMS Microbiology Reviews* 29 (2005) 625-651.
- [50] P.L. Conway, S.L. Gorbach, B.R. Goldin, Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells, *Journal of Dairy Sciences* 70 (1) (1987) 1-12.
- [51] M. Du Toit, C.M. Franz, L.M. Dicks, U. Schillinger, P. Haberer, B. Warlies, et al., Characterisation and selection of probiotic lactobacilli for a preliminary mini pig feeding trial and their effect on serum cholesterol levels, faeces pH and faeces moisture content, *International Journal of Food Microbiology* 40 (1998) 93-104.
- [52] C.N. Jacobsen, V. Rosenfeldt Nielsen, A.E. Hayford, P.L. Moller, K.F. Michaelsen, A. Paerregaard, et al., Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by *in vitro* techniques and evaluation of the colonization ability of five selected strains in humans, *Applied Environment Microbiology* 65 (1999) 4949-4956.
- [53] C. Dunne, L. Murphy, S. Flyin, L. O'Mahony, S. O'Halloran, M. Feeney, et al., Probiotics: From myth to reality, demonstration of functionality in animal models of disease and in human clinical trials, *Antonie van Leeuwenhoek* 76 (1999) 279-292.
- [54] V. Xanthopoulos, E. Litopoulou-Tzanetaki, N. Tzanetakis, Characterization of *Lactobacillus* isolates from infant faeces as dietary adjuncts, *Food Microbiology* 17 (2) (2000) 205-215.
- [55] J.H. Park, J.I. Um, B.J. Lee, J.S. Goh, S.Y. Park, W.S. Kim, et al., Encapsulated *Bifidobacterium bifidum* potentiates intestinal IgA production, *Cellular Immunology* 219 (2002) 22-27.
- [56] A. Fernández, J.L. Ruiz-Barba, Optimization of bacteriocin production by batch fermentation of *Lactobacillus plantarum* LPCO10, *Applied Environmental Microbiology* 68 (2002) 4465-4471.
- [57] P. Marteau, M. Minekus, R. Havenaar, J.H.J. Huis In't Veld, Survival of lactic acid bacteria in a dynamic model

- of the stomach and small intestine: Validation and the effects of bile, *Journal of Dairy Sciences* 80 (6) (1997) 1031-1037.
- [58] J. Prasad, H. Gill, J. Smart, P.K. Gopal, Selection and characterisation of *Lactobacillus* and *Bifidobacterium* strains for use as probiotics, *International Dairy Journal* 8 (12) (1998) 993-1002.
- [59] T. Kalambaheti, G.N. Cooper, G.D.F. Jackson, Role of bile in non-specific defence mechanisms of the gut, *Gut* 35 (1994) 1047-1052.
- [60] C. Dunne, L. O'Mahony, L. Murphy, G. Thornton, D. Morrissey, S. O'Halloran, et al., *In vitro* selection criteria for probiotic bacteria of human origin: Correlation with *in vivo* findings, *Journal of Clinical Nutrition* 73 (2) (2001) 386-392.
- [61] M.E. Sanders, D.C. Walker, K.M. Walker, K. Aoyama, T.R. Klaenhammer, Performance of commercial cultures in fluid milk applications, *Journal of Dairy Sciences* 79 (6) (1996) 943-955.
- [62] M.G. Gänzle, C. Hertel, J.M. van der Vossen, W.P. Hammes, Effect of bacteriocin-producing lactobacilli on the survival of *Escherichia coli* and *Listeria* in a dynamic model of the stomach and the small intestine, *International Journal of Food Microbiology* 48 (1) (1999b) 21-35.
- [63] M. Begley, G.G. Gahan, C. Hill, Bile salt hydrolase activity in probiotics, *Applied Environment Microbiology* 72 (2006) 1729-1738.
- [64] M.T. Liong, N.P. Shah, Acid and bile tolerance and cholesterol removal ability of Lactobacilli strains, *Journal of Dairy Science* 88 (2005) 55-66.
- [65] S. Gilliland, T. Staley, L. Bush, Importance of bile tolerance of *Lactobacillus acidophilus* used as dietary adjunct, *Journal of Dairy Science* 67 (1984) 3045-3051.
- [66] M.P. Taranto, F. Sesma, A. Pesce de Ruiz Holgado, G.F. de Valdez, Bile salts hydrolase plays a key role on cholesterol removal by *Lactobacillus*, *Biotechnology Letters* 19 (9) (1997) 845-847.
- [67] N. Sridevi, P. Vishwe, A. Prabhune, Hypocholesteremic effect of bile salt hydrolase from *Lactobacillus buchneri* ATCC 4005, *Food Research International* 42 (2009) 516-520.
- [68] C.M. Franz, M. Huch, H. Abriouel, W. Holzapfel, A. Gálvez, Enterococci as probiotics and their implications in food safety, *International Journal of Food Microbiology* 151 (2) (2011) 125-140.
- [69] B.Z. De Rodas, S.E. Gilliland, C.V. Maxwell, Hypocholesterolemic action of *Lactobacillus acidophilus* ATCC 43121 and calcium in swine with hypocholesterolemia induced by diet, *Journal of Dairy Science* 79 (1996) 2121-2128.
- [70] F.M. Driessen, R. De Boer, Fermented milks with selected intestinal bacteria: A healthy trend in new products, *Netherland Milk Dairy Journal* 43(1989) 367-382.
- [71] F. Berr, G.A. Kullak-Ublick, G. Paumgartner, W. Munzing, P.B. Hylemon, W.P. Charteris, et al., Effect of conjugated bile salts on antibiotic susceptibility of bile salt-tolerant *Lactobacillus* and *Bifidobacterium* isolates, *Journal of Food Protection* 63 (1996) 1369-1376.
- [72] A. Mamianett, D. Garrido, C.N. Carducci, M.C. Vescina, Fecal bile acid excretion pattern profile in gallstone patients, *Medicina* 59 (1999) 269-273.
- [73] Y.T. Ahn, G.B. Kim, K.S. Lim, Y.J. Baek, H.U. Kim, Deconjugation of bile salts by *Lactobacillus acidophilus* isolates, *International Dairy Journal* 13 (4) (2003) 303-311.
- [74] S.E. Gilliland, M.L. Speck, Use of the Ylinitex system for characterizing lactobacilli, *Applied Environment Microbiology* 33 (1977) 1289-1292.
- [75] T. Takahashi, M. Morotomi, Absence of cholic acid 7 α -dehydroxylase activity in the strains of *Lactobacillus* and *Bifidobacterium*, *Journal of Dairy Sciences* 77 (1994) 3275-3286.
- [76] L.A. Thomas, M.J. Veysey, T. Bathgate, A. King, G. French, N.C. Smeeton, et al., Mechanism for the transit-induced increase in colonic deoxycholic acid formation in cholesterol cholelithiasis, *Gastroenterology* 199 (2000) 806-815.
- [77] M.J. Veysey, L.A. Thomas, A.I. Mallet, P.J. Jenkins, G.M. Besser, G.M. Murphy, et al., Colonic transit influences deoxycholic acid kinetics, *Gastroenterology* 121 (2001) 812-822.