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Screening for Novel Probiotic Candidate: Survival, Antimicrobial, and Aggregation Properties of *Lactobacillus* Strains from Moroccan Dairy Products

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Abstract

In view of the growing interest in dairy probiotic foods, and in order to increase the knowledge on the biodiversity for their possible inclusion as probiotics strains in microbiology applied to the food industry. This study aims to assess the probiotic potential of 17 *Lactobacillus* strains isolated from local dairy products: fresh cheese and fermented milk. First PCR amplification of repetitive bacterial DNA fingerprints (rep-PCR) using the ERIC primers allowed the *Lactobacillus* strains to be grouped into the species: *L. plantarum*, *L. casei, L. paracasei, L. curvatus*, and *L. rhamnosus and* showed a considerable degree of genomic diversity. We evaluated Lactobacillus strains for sustained cell viability under different conditions similar to the environment of the intestinal tract. We also assessed aggregation properties and antimicrobial activity. The results of the *in vitro* assessment demonstrated that *lactobacillus* strains did not show the presence of any genes encoding virulence factors, also *L. plantarum,* and *L. rhamnosus* remained functionally active under these experimental conditions, with an excellent survival capacity strain under low pH and high bile salt concentration, a good antimicrobial activity and a good capacity to auto-aggregate with each

other's, and co-aggregate with pathogenic bacteria. Based on the data, we suggest the isolated *L. plantarum,* and *L. rhamnosus* isolated from the traditional dairy product may be a probiotic candidate for *in vivo* behavioral.

Keywords: Dairy products, Rep-PCR, Probiotics, Stress conditions, Proteolytic activity, Lipolytic activity

Practical Application

Lactobacillus strains isolated from local fresh cheese and fermented milk have shown a high level of genetic diversity. *L. plantarum, and L. rhamnosus* were found to have an excellent survival capacity under low pH and high bile salt concentration, a good antimicrobial activity, and a good capacity to auto-aggregate with each other's, and co-aggregate with pathogenic bacteria. This finding would provide scientific support to wide consumption of the original traditionally Moroccan dairy products and health community will come true. And in turn open an opportunity the development or expanding of food industry.

Introduction

In Morocco, dairy production of dairy cows is of great importance in agriculture and plays a fundamental role in feeding a growing and increasingly urban population. Milk production increased from 475 million litres in 1975 to 1 billion 331 million litres in 2002 [1]. Dairy products made from locally produced raw milk remain a very important part of the daily diet. People living in the country use milk to produce "jben" of cottage cheese, "smen" of fermented butter and "lben" of fermented skim milk [1].

In the north of Morocco, the fermented milk, '*lben*' is traditionally made from raw cow milk by spontaneous fermentation. Many Moroccan traditional dairy products have a good nutritional value that merit production at small and large industrial scale to standardize their processing stages, improve their safety and shelf life, and enhance their added value properties. Thus, officially recognizing these dairy products and allowing them to be formally marketed could, in turn, contribute to the sustainable development of the countryside. Unfortunately, only a few products have been scientifically studied to allow standardization of their technological developments [2]. The stability of the microbial content of these products over time is not well known. However, environmental conditions such as temperature, origin and quality of the milk, processing and sanitary conditions, might have a significant influence on the microbial composition of traditionally made dairy products [3].

Lactic acid bacteria (LAB) are among the most important groups of microorganisms in food fermentation. They contribute to the taste and texture of fermented products, and their presence is correlated with their role as starter cultures and bio-preservatives [4]. In addition, they inhibit food spoilage and pathogenic bacteria by producing an array of antimicrobial substances. Although the latest research shows that *Lactobacillus* strains open new possibilities for controlling *L. monocytogenes* by biofilms formation as a "natural" immobilization way dispersal, whether species interactions could modify the virulence *of L. monocytogenes* still remains unclear [5].

LAB probiotic potential is extensively studied due to the ability of these bacteria to tolerate the gastrointestinal conditions and their potential appliance on food industries as probiotic cultures [6]. LAB are widely used in the food industry as probiotics [7]. Probiotics include "good and live microorganisms" that benefit the host's health when administered in adequate amounts (FAO/WHO, 2001). Among them, bacteria, especially LAB–mainly represented by *Bifidobacterium* and *Lactobacillus,* are the most used probiotics besides yeasts [8]. Probiotic microorganisms should overcome the physical and chemical barriers, which include the acid and bile stress, as well as pancreatic enzymes during the gastrointestinal transition. The adhesion of probiotic microorganisms to the intestinal epithelium is essential for the colonization of the gastrointestinal tract epithelium. It plays a vital role in preventing bacterial elimination by peristalsis and providing a competitive advantage in the ecosystem [9]. Probiotic microorganisms that can co-aggregate with pathogens may be more efficient in eliminating undesirable bacteria because of antimicrobial substances production in very close proximity [10].

Considering the commercial interest for novel potential probiotic strains and the lack of knowledge about the LAB in traditional Moroccan dairy products, the finding would provide scientific support to wide consumption of the original traditionally Moroccan dairy products and health community will come true. And in turn open an opportunity the development or expanding of food industry.

This study aims to identify potential probiotic strains isolated from traditional fresh cheese "Jben" and fermented milk "Lben" and characterize the transcriptional response of selected probiotic strains in acid stress Several biological properties like antimicrobial, aggregation properties were investigated to select strains with relevant industrial traits for possible use as probiotic strains.

Materials and Methods

Sampling and isolation of LAB

Seventeen *Lactobacillus strains* isolated from traditional fresh cheese and fermented milk were purchased from the rural markets of Tangier, North of Morocco. The samples were collected in sterile bottles and kept at 4°C until arrival at the laboratory. Samples were serially diluted in sterile saline solution and plated on de Man, Rogosa and Sharpe (MRS) agar in triplicate. After two days of incubation under aerobic conditions at 30°C, colonies were counted and isolated. The isolates were randomly picked from MRS agar plates, purified, and assessed for catalase activity and Gram reaction. Only Gram-positive, catalase-negative isolates were selected and stored at -20°C in liquid culture using 25% glycerol.

Phenotypic characterization of isolated strains

Lactobacillus strains were identified phenotypically to the genus level, as described by Yousif et al. [11]. Criteria followed for the phenotypic identification consists the following tests: growth at different temperatures (10, 30, 40, 45 and 50°C), varying concentrations of NaCl (4, 6.5, and 8%, w/v), the determination of the fermentation type by the production of gases (CO_2) and lactic acid, and hydrolysis of arginine. Hydrolysis of arginine was detected by the production of arginine dihydrolase (ADH) in the M16 BCP medium with sodium bicarbonate and sodium lactate, Sigma-Aldrich, USA.

Genotypic characterization

PCR identification of LAB isolates: Phenotypically identified strains of *Lactobacilli, and Enterococcus were* confirmed by PCR using specific primers (Table 1). The total DNA for PCR reactions was obtained by a freeze/unfreeze shock method, and the Lysed crude extracts were stored at -20°C until being used as templates (1.2 µL). The PCR primers employed in the present study were synthesized by *Invitrogen™ Custom* DNA Oligos (Life Technologies Corporation, Carlsbad, CA, USA). In brief, PCR was performed in a 15 µL mixture containing 1.2 µL DNA template, 50 Pm of each primer, 0.2 mM of each deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 75 mM Tris–HCl, 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20 and 0.625 units of Thermoprime Taq DNA polymerase (2× ReadyMix TM PCR Master Mix, ABgene, UK). The PCR was performed in a DNA-Thermal cycler (BioRad, Applied Biosystems, CA, USA) using different programs to ensure experimental specificity (Table 1). The PCR-products were resolved by electrophoresis on agarose gel (2 g/100 mL) containing 0.5 g/mL ethidium bromide at 120 V.

rep-PCR and electrophoresis: The rapid and reproducible genotypic technique (rep-PCR) was carried out to the strains for there could be reliably identified at the (sub)species-level and assess the genetic relationship of the lactobacillus strains and also using methods previously described by Gevers et al. [12]. The rep-PCR oligonucleotide primers evaluated in this study were:
ERIC1 [5'ATGTAAGCTCCTGGGGATTCAC-3'];

ERIC2 [5'AAGTAAGTGACTGGGGTGAGCG-3'] (Sigma - Aldrich, USA) Amplification reactions were prepared in a total volume of 25 µL per tube, comprising:

100 ng genomic DNA, 10 pmol of each primer (1x buffer, 200 µM of each deoxyribonucleotide triphosphate (Promega, USA), 1.5 mM $MgCl_2$ and 1.0 U Taq DNA polymerase (Promega, USA). PCR conditions described by Versalovic et al, 1994 used were: 95°C for 3 min, followed by 30 cycles of denaturation at 92°C for 1 min, annealing at 36°C for 1 min and extension at 72°C for 8 min, followed by a final extension at 72°C for 16 min. agarose gel

Table 1: PCR primers used in this study.

Genus/species	Primers	Sequence 5'-3'	Thermal cycler program	References
L. paracasei	Y2 Para	CCCACTgCTgCCTCCCgTAggAgT CACCgAgATTCAACATgg	94°C for 3 min 30 cycles: •45°C for 1 min •72°C for 30s •94°C for 45s 72°C for 1min	Ward and Timmins [42]
L. plantarum	P16 LP1	GCTggATCACCTCCTTTC ATgAggTATTCAACTTATg	94°C for 5 min 30 cycles: •94°C for 1 min •53°C for 30s •72°C for 1 min 72°C for 7min	Berthier and Ehrlich [43]
L. casei	LSC LBLR2	gggggATAACATTT CCATgCACCACCTgTCATTTT	92°C for 5 min 30 cycles: •92°C for 30s •56°C for 30s •72°C for 1 min 72°C for 10min	Roy, et al. [44]
L. rhamnosus	Rha2 PCI	gCgATgCgAATTTCTATTATT CAgACTgAAAgTCTgACGG	92°C for 2 min 30 cycles: •95°C for 30 s •58°C for 30s •72°C for 30 s 72°C for 1min	Walter, et al.
L. curvatus	LcurvR 16FOR	TTggTACTATTTAATTCTTAG gCTggATCACCTCCTTTC	94°C for 5 min 30 cycles: •94°C for 30 s •58°C for 30s •72°C for 1min 72°C for 7min	Berthier and Ehrlich [43]
Lactobacillus spp	LbLMA1-rev R16-1	CTCAAAACTAACAAAgTTTC CTTgTACACACCgCCCgTCA	95°C for 5 min 30 cycles: •95°C for 30 s •55°C for 30s •72°C for 2 min 72°C for 7min	Dubernet, et al. [45]
Enterococcus Spp	Ent 1 Ent 2	TACTgACAAACCATTCATgATg AACTTCgTCACCAACgCgAAC	95°C for 3 min 35 cycles: •95°C for 30 s •45°C for 30s •75°C for 2 min 72°C for 7min	Ke, et al. [46]

electrophoresis of PCR product was done according to Gevers et al. [12]. PCR product were stained with blue-green dye (LGC Biotecnologia, Brazil) and subjected to electrophoresis on 1.5% agarose gel in Tris-borate-EDTA (TBE) buffer under constant voltage of 100V for 3 h. The sizes of the bands were determined using a molecular weight marker (Hyper Ladder™), ranging from 50 bp to 2000 bp (Bioline, USA) (Figure 2-4). Obtained ERIC-PCR fingerprints were normalized using the BioNumerics software (version 7.6) (Applied Maths, Sint-Martens-Latem, Belgium). Groupings of the rep-PCR fingerprints were performed using the Pearson's correlation coefficient (r) and the unweighted pair group method using arithmetic averages clustering algorithm (UPGMA).

Biotechnological potential of LAB

Antimicrobial activity: The wells were impregnated with 60 to 80 µL of filtered and neutralized supernatant obtained after centrifugation at 4,000 rpm for 15 min from a culture of the *Lactobacillus strains* [13].

Mueller-Hinton blood agar medium (1 mL) was poured into sterile Petri dishes and allowed to solidify. The boxes were seeded on the surface by the suspension of the pathogenic strain (*Enterococcus faecalis* ATCC 19433, *Listeria monocytogenes* ATCC 19144, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Salmonella thyphimurium* ATCC 14028, and *Pseudomonas seruginosa* ATCC 27853). The petri dishes were placed on the culture medium and incubated at 37°C for 24 h for the diffusion of antimicrobial agents. The diameters of the inhibition zones around the discs were measured, and the diameters greater than 2 mm was considered positive for antimicrobial activity. The inhibition diameter (I.D.) was measured using Equation (1):

ID (mm) = diameter of inhibition zone obtained (mm) – diameter of well (5 mm) (Eq.1)

Proteolytic and lipolytic activity: The study consisted of assessing enzymes of biotechnological potential such as proteolytic enzymes by the method described by Gordon et al. [14]. The proteolytic activity of LAB was highlighted and compared on 1, 3, and 5% (w/v) milk agar. The young bacterial culture was seeded on the surface of milk agar and incubated at 30°C. The proteolytic activity was manifested by the appearance of a clear halo around the colonies.

The lipolytic activity of LAB was evaluated on a solid (MRS) medium buffered at pH 7 (phosphate buffer Na_2HPO_4/NaH_2PO_4, 0.2 M) containing concentrations of 1%, 3%, and 5% Tween 80 as the sole lipid source, 0.01% (w/v) calcium chloride ($CaCl_2$) and 0.5% (w/v) sodium chloride (NaCl). Lactic strains are seeded on the medium and incubated at 30°C for seven days. Lipolytic activity was observed by the appearance of a halo opaque around the colony [15].

Acidifying power and coagulation time: The acidification and the coagulation time were evaluated for all the *Lactobacillus* strains. The acidification was assessed by monitoring acidity Dronic of LAB strains incubated at 37°C during 0 h, 2 h, 6 h, 24 h, and 30 h, and the coagulation time was noted once coagulation was observed [16].

Survival capacity: bile salts and acid tolerance: Seventeen *Lactobacillus* spp, Gram-positive, catalase-negative isolates. Selected according to their biological properties and antimicrobial activity were tested for survival at low pH (1.5 and 2) and bile salt tolerance (1%, 2%, 3%, and 4%) [17]. MRS agar with varying concentrations of bile salt mixture (Sigma B-3426, US) was inoculated onto the surface by overnight grown MRS broth cultures (100 mL). The plates were incubated at 37°C for 72 h and visually observed for bacterial growth as a lawn, indicating resistance to the specific concentration of bile salt.

For determination of acid tolerance (low pH), overnight grown MRS broth cultures (1 mL) were inoculated onto 19 mL simulated gastric fluid (3.2 g/L pepsin and 2 g/L NaCl) adjusted at different pH values with 5M HCl. After incubation for 30 min at 37°C, 1 mL mixture was used to determine viable counts (expressed as cfu mL^{-1}) on MRS agar, using the concentration of viable bacteria grown without simulated gastric fluid as the reference [17]. The percentage survival of the bacteria in low pH was calculated as follows equation (2):

% Survival = log cfu of viable cells survived / log cfu of initial

viable cells inoculated × 100. *(Eq.2)*

Security aspect: DNAse activitie were determined as described by Lavilla-Lerma et al. [18] DNAse activity was determined by spot inoculating the culture (5 µL) onto DNAse agar plates (Scharlau, Spain). The plates were overlaid with 1 N HCl for 5 min after incubation at 37°C for 48 h. DNA hydrolysis test or Deoxyribonuclease (Dnase) test is used to determine the ability of an organism to hydrolyze DNA and utilize it as a source of carbon and energy for growth. If the organism that grows in the medium produces Deoxyribonuclease, it breaks down DNA into smaller fragments. When the DNA is broken down, it no longer binds to the methyl green, and green color fades and the colony is surrounded by a colorless zone. Gelatinase production was evaluated by inoculating the strains (5 µL) on Butylated hydroxyanisole (BHA) supplemented with 0.04% gelatine and incubated at 37°C for 24 h followed by for 20 min at 4°C; then, the gelatinase activity was assessed by analyzing media consistence [19]. These experiments were performed with two biological replicates. *S. aureus* ATCC 25923 was used as a positive control for both tests.

Aggregation properties

Auto-aggregation: Overnight grown *Lactobacilli* strains in MRS broth was harvested (2 mL), washed, and resuspended in sterile phosphate-buffered saline solution (PBS). After 2 h at room temperature, 100 µL was removed from the aqueous phase of the suspension and transferred to 900 µL of PBS and the absorbance (A) was measured at 580 nm [20]. The percentage of auto-aggregation was expressed in equation (3) where A_t absorbance at time t = 2h and A_0 absorbance at time t = 0 h.

Agg% = [1 - (A_t of upper suspension / A_0 of total bacterial suspension)] × 100 (Eq.3)

Co-aggregation: The ability of the co-aggregation of *Lactobacillus* with pathogenic bacteria (*Listeria monocytogenes* ATCC 19144, *Escherichia coli CCUG* 47553) was performed according to Vlková et al. [21]. *Lactobacillus* and the pathogenic bacteria (*Listeria monocytogenes* and *E. coli*) were grown separately on Trypticase phytone yeast extract broth (TPY) (Sharlau, Spain) and Reinforced Clostridial Medium (RCM) broth (Oxoid, UK), respectively. The cultures were mixed, and co-aggregation tests were carried out by observing under the dark field microscope (Eclipse E800 biological research microscope, Nikon). Each experiment was conducted twice and pure cultures were used as controls. The percentage of coaggregation was determined using the equation (4):

$$\% \text{ Coaggregation} = [(A_{\text{test bacteria}} + A_{\text{lactic acid bacteria}}) - 2 \times (A_{\text{mixed strains}}) / (A_{\text{test bacteria}} + A_{\text{lactic acid bacteria}})] \times 100 \text{ (Eq.4)}$$

Biofilm formation: The formation of biofilm by *Lactobacillus* was assessed as described by Toledo-Arana et al. [22]. Briefly, *Lactobacillus* strains were grown overnight at TSB with 0.25% glucose at 37°C. Culture was diluted at 1:40 in 0.25% of the TSB and 200 µl of this cell suspension were used to inoculate sterile 96-well polystyrene microtiter plates. After 24 hours at 37°C, the wells were gently washed three times with 200 µl phosphate buffered saline (PBS), dried in the inverted position and stained with 1% (w/v) crystalline violet for 15 min. The wells were rinsed again, and the violet crystal was solubilized in 200 µl of ethanol-acetone (80:20, v/v). The optical density at 595 nm (OD595) was determined using a microplate reader (Varioskan Flash Reader, Thermo Scientific, USA) using 1% crystalline violet. Each test was carried out in triplicate and repeated three times.

Statistical analysis

All experiments were performed in triplicate using (Excel 2007, Microsoft 365. USA) to determine the average and standard deviations and presented as mean ± standard deviation (absolute). Statistical treatment of adhesion data was conducted by analysis of variances (ANOVA 1) in Statgraphics Centurion XVI software using Shapiro–Wilk test and the Levene test to check data normality. The two-sided Tukey's test was used to determine the significance of differences between strains. Statistical significance was determined at $P < 0.05$.

Results and Discussion

Genotypic identification by PCR

The isolated lactic bacteria were first assigned at the genus and species level by PCR 16s with the use of specific primer pairs (rRNA 16s) (Table 1). PCR genotypic characterization shows

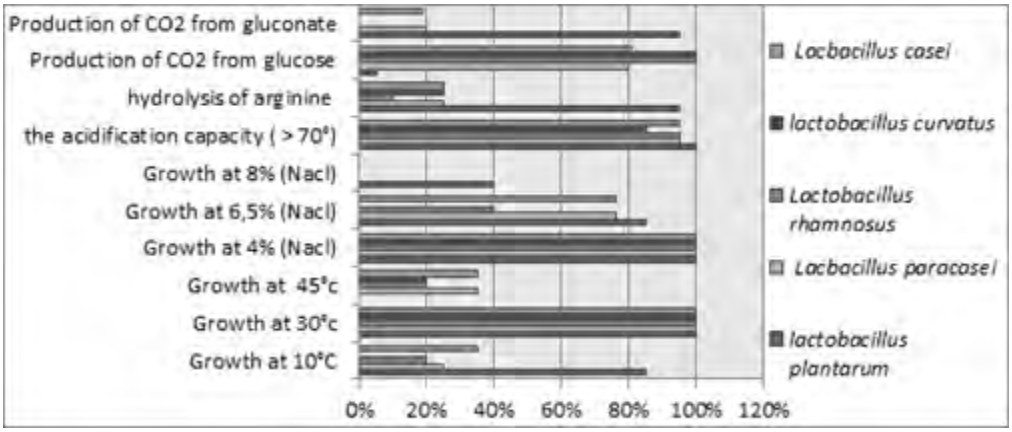

Figure 1: Phenotypic characterization of lactobacillus strains isolated from traditional cheeses "JBEN" and fermented milk "LBEN".

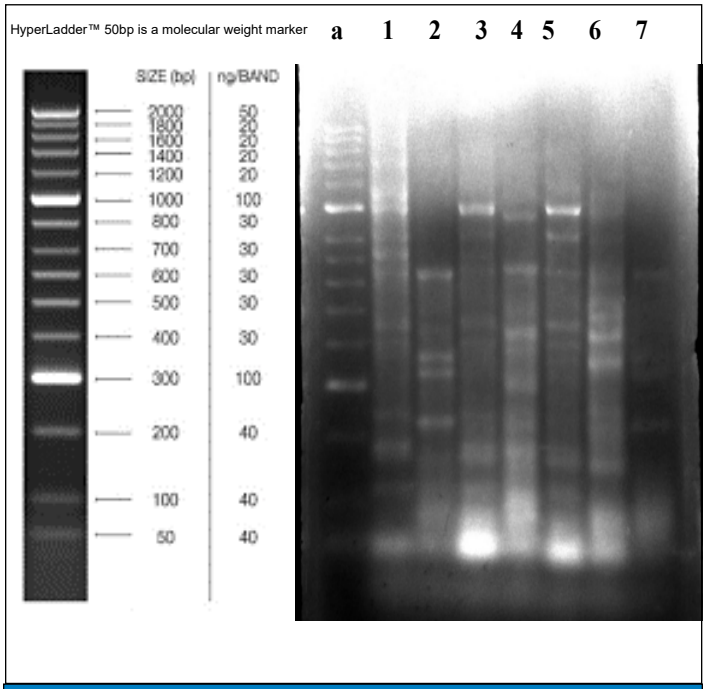

Figure 2: Agarose gel electrophoresis of rep-PCR products (for LAB isolated from fresh cheese "jben"). lane (a) DNA marker (Hyperladder™ 50bp, Bioline) with 50 bp to 2000 bp scale and 300 bp, 1000 bp and 2000 bp high reference bands; lane 1 *L. plantarum* **1, lane 2** *L. plantarum* **5, lane 3** *L. casei* **7a, lane 4** *L. paracasei* **10, lane 5** *L. plantarum* **11, lane 6** *L. plantarum* **12, lane 7** *L. plantarum* **21.**

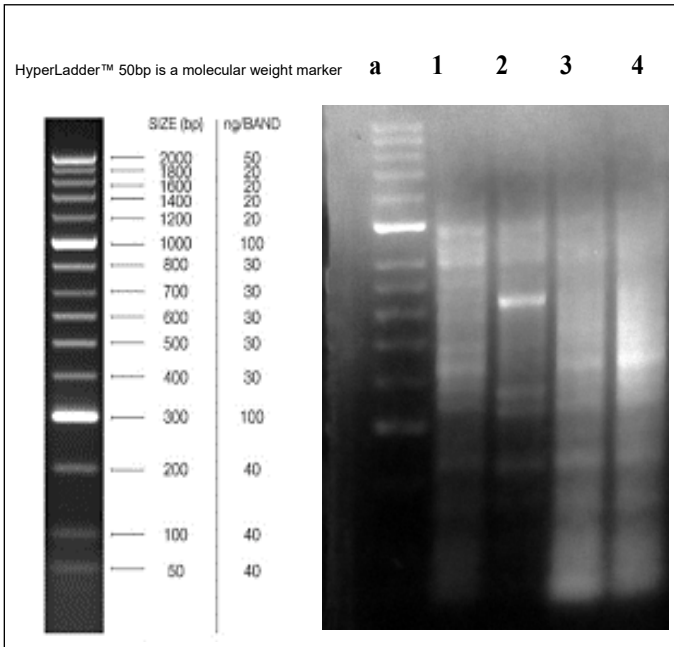

Figure 3: Electrophorese agarose gel products from rep-PCR products (for LAB isolated from fresh cheese "jben"). Lane (a) DNA marker DNA marker (Hyperladder™ 50bp, Bioline) with 50 bp to 2000 bp scale and 300 bp, 1000 bp and 2000 bp high reference bands; lane 1 *L. paracasei* **3, lane 2,** *L. paracasei* **54, lane 3,** *L. paracasei* **137, lane 4,** *L. plantarum* **1.**

Table 2: Species and corresponding origin of Lactobacillus strains.

Strain	Code	Origine
L. casei 7a	L. casei 7a	Jben: traditional fresh cheese
L. paracasei 54	L.para 54	Lben: traditional fermented milk
L. plantarum 1	L. pl 1	Lben: traditional fermented milk
L. paracasei 3	L. para 3	Lben: traditional fermented milk
L. plantarum 5	L. pl 5	Jben: traditional fresh cheese
L. plantarum 11	L. pl 11	Jben: traditional fresh cheese
L. plantarum 12	L. pl 12	Jben: traditional fresh cheese
L. plantarum 21	L. pl 21	Jben: traditional fresh cheese
L. plantarum 53	L. pl 53	Lben: traditional fermented milk
L. plantarum 57	L. pl 57	Lben: traditional fermented milk
L. plantarum 62	L. pl 62	Lben: traditional fermented milk
L. plantarum 63	L. pl 63	Lben: traditional fermented milk
L. curavtus 81	L. pl 1	Lben: traditional fermented milk
L. rhamnosus 91	L. rh 91	Lben: traditional fermented milk
L. plantarum 108	L. pl 108	Lben: traditional fermented milk
L. curvatus 134	L. cur 134	Lben: traditional fermented milk
L. paracasei 137	L. para 137	Jben: traditional fresh cheese
L. paracasei 10	L. para 10	Jben: traditional fresh cheese

that: the 17 bacillus-shaped lactic strains belong to the genus *Lactobacillus spp* of which: 9 are *Lactobacillus plantarum*, four of them are *Lactobacillus paracasei*, one is *Lactobacillus rhamnosus*, and two of them are *Lactobacillus curvatus*. Among these 17 strains studied, 10 were, from fresh cheese "Jben" and 7 were from fermented milk "Lben" (Table 2).

rep-PCR appears to have the potential to distinguish between the *Lactobacillus* and other related bacterial species in the food industry to produce precise results. Overall, rep-PCR is an easy, rapid, reliable, and reproducible molecular typing method that can provide species-specific information. The LAB strains were characterized further using rep-PCR, and the sizes of the bands were determined using a molecular weight marker ranging from 50 bp to 2000 bp (Bioline, USA) [12]. The results of gel electrophoresis banding profiles with the ERIC-PCR showed an average of 17 bands for the *L. casei* 7a, *L. paracasei* 10, *L. plantarum* (1, 5, 11, 12 and 21) strains (Figure 2). The largest band (200 and 300 bp) was observed for all the *Lactobacillus* strains (isolated from fresh cheese). A prominent band of 1000 bp was reported in all the isolates except *L plantarum* 5, *L. plantarum* 21. In addition, *L. plantarum* 21 (lane 7) consisted of six (light single band) bands (single light band) of 600, 400, 300, 200, 100, and 50 bp. Moreover, the ERIC-PCR generated profiles for the *L. plantarum* 1, and *L. paracasei* (3, 54, 137) strains with an average of 20 bands (Figure 3). The largest bands (200 and 300 bp) were observed for all *Lactobacillus* strains (isolated from fresh cheese), and the prominent band of 1000 bp was reported in all the isolates. On the other hand, ERIC-PCR generated profiles with an average of 12 bands (lane 1 and 7) for *L. curvatus* 81, and *L. plantarum* 63 and six bands for *L. curvatus* 134, *L. rhamnosus* 91, *L. plantarum* (108, 62 and 57) (lanes 2, 3, 4, 5, and 6) (isolated from traditional fermented milk) (Figure 4). The largest (200, 300 bp) and prominent band (1000 bp) were observed for all *Lactobacillus* strains except *L. curvatus* 134 and *L. rhamnosus* 91(lane 1 and 7). Also, the prominent band was not observed for *L. plantarum* 108. A unique noticeable band (50 bp) was represented by only *L. curvatus* 134 (lane 1). A unique banding profile consisting of 500 bp (light band), 400, 200,100, and 50 bp was reported for *L. rhamnosus* 91 (lane 3).

The rep-PCR shows a great genetic diversity that differs from one species to another and from one product to another, in general the strains of lactobacillus from fresh cheese were able to show a great diversity (20 and 17 bands) unlike strains from fermented milk (12 bands). Also *L. plantarum* could show different profiles (20, 17 and 6 bands) which reflects its remarkable genetic diversity. Confirming the high genetic diversity *of L. plantarum* previously reported by other authors [23,24].

Phenotypic characterization of isolated strains

The seventeen isolated strains (from traditional cheese and fermented milk) were identified as *lactobacillus spp*. The phenotypic characterization of LAB strains showed different properties depending on the species, which are essential for carrying out the fermentation process (Figure 1).

In the present study, 9 LAB strains with rod-shaped morphology

Table 3: Antibacterial activity of Lactobacillus strains (n=17).

Strains	E coli	Enterococcus fecalis	Staphilococcus aurus	Salmonella thyphimurium	Listeria monocytogenes	Pseudomonas seruginosa
	ATCC 25922	ATCC 19433	ATCC 25923	ATCC 14028	ATCC 19144	ATCC 27853
	Inhibition zone expressed (mm)					
L. casei 7a	6±0.67^{a}	12±0.35^{b}	12±0.51^{b}	7±0.55^{a}	12±0.55^{b}	8±0.57^{a}
L. paracasei 54	7±0.50^{a}	7±0.87^{a}	6±0.47^{a}	7±0.74^{a}	11±0.50^{ab}	6±0.35^{a}
L. plantarum 1	7±0.53^{a}	6±0.67^{a}	8±0.56^{a}	6±0.50^{a}	8±0.75^{a}	8±0.33^{a}
L. paracasei 3	6±0.85^{a}	6±0.50^{a}	8±0.43^{a}	8±0.34^{a}	8±0.35^{a}	6±0.56^{a}
L. plantarum 5	6±0.55^{a}	6±0.45^{a}	8±0.67^{a}	6±0.56^{a}	8±0.79^{a}	6±0.56^{a}
L. plantarum 12	14±0.34^{b}	6±0.50^{aa}	6±0.55^{a}	6±0.67^{a}	6±0.53^{a}	8±0.67^{a}
L. plantarum 21	14±0.65^{b}	9±0.73^{ab}	8±0.35^{a}	6±0.74^{a}	5±0.67^{a}	8±0.54^{a}
L. plantarum 53	6±0.85^{a}	6±0.50^{a}	8±0.43^{a}	8±0.34^{a}	8±0.35^{a}	6±0.56^{a}
L. plantarum 57	6±0.50^{a}	6±0.78^{a}	6±0.61^{a}	5±0.53^{a}	5±0.54^{a}	8±0.65^{a}
L. plantarum 62	6±0.45^{a}	6±0.47^{a}	6±0.75^{a}	8±0.45^{a}	5±0.50^{a}	8±0.50^{a}
L. plantarum 63	14±0.53^{b}	6±0.89^{a}	6±0.45^{a}	8±0.65^{a}	5±0.55^{a}	5±0.73^{a}
L. curavtus 81	8±0.85^{a}	6±0.45^{a}	6±0.89^{a}	8±0.50^{a}	7±0.87^{a}	5±0.79^{a}
L. rhamnosus 91	6±0.35^{a}	12±0.75^{b}	12±0.45^{b}	8±0.65^{a}	7±0.67^{a}	5±0.68^{a}
L. plantarum 108	8±0.75^{a}	5±0.76^{a}	8±0,54^{a}	12±0,59^{b}	8±0,69^{a}	5±0,35^{a}
L. curvatus 134	8±0.53^{a}	5±0.48^{a}	5±0,74^{a}	5±0,67^{a}	5±0,45^{a}	8±0,55^{a}
L. paracasei 137	6±0.89^{a}	6±0.67^{a}	6±0,53^{a}	6±0,32^{a}	5±0,47^{a}	5±0,35^{a}
L. paracasei 10	6±0.65^{a}	6±0.53^{a}	6±0,45^{a}	6±0,54^{a}	8±0,67^{a}	6±0,75^{a}

*Values are expressed as the mean ± SD of three independent experiments (n = 3). *Different lower-case letters represent significant differences between strains (P < 0.05).*

belonging to the family of *L. plantarum* were not able to produce CO_2 from glucose, indicating they belonged to homo-fermentative LAB groups. This LAB group was only able to ferment glucose to lactic acid. Conversely, 17 rod-shaped LAB strains produced gas from glucose fermentation, classified as obligate hetero-fermentative. However, *L. paracasei, L. casei, L. rhamnosus,* and *L. curvatus* belonging to the hetero-fermentative LAB group, were able to ferment glucose to lactic acid, ethanol / acetic acid, and CO_2 [25]. It was found that only *L. plantarum* was able to hydrolyze arginine; however, less than 25% of the remaining *Lactobacillus* strains produced arginine dihydrolase (ADH).

Temperature is a crucial factor in bacterial growth. In this study, the isolated *Lactobacillus* spp. were able to grow within 10 - 45°C, and the optimum temperature was 37°C. *L. plantarum* showed growth at 10°C, but no growth at 45°C. In contrast, *L. paracasei*, *L. casei*, *L. rhamnosus,* and *L. curvatus* could grow at 45°C; however, less than 35% of strains were able to grow at 10°C. Moreover, all *Lactobacillus* strains could grow at 4% NaCl (Figure 1), and only 45% of *L. plantarum* could grow at 8%. The results were concordant with findings of Hoque, (2010), who reported that *Lactobacillus* spp. were resistant to inhibitory substances such as NaCl (1 - 9%).

Dendrogram analysis

The dendrogram was obtained by the unweighted neighbor-joining method using genetic dissimilarities based on rep-PCR data (Figure 5). The seventeen strains were divided into three main groups consisting of Ten and six strains using the ERIC-PCR. Group I was subdivided into six strains of *lactobocillus* in which are found: *L. casei* 7a, *L. paracasei* 10 and *L. plantarum* (5, 11, 21and 12) with a percentage of similarity of 67.3% The fact that strains of *L. casei 7a*, and *L. paracasei* 10 have the same band profile observed *L. plantarum* is probably due to genetic similarities of *lactobacillus* strains isolated all from traditional

cheese (Figure 5). Group II consists of ten strains of *lactobacilli* which showed interesting genetic similarities of 69,2%: *L. curvatus* 81, *L. paracasei* 54, *L planatrum* 1, *L. plantarum* 57, *L. plantarum* 108, *L. plantarum* 62, *L. curvatus* 134, *L. plantarum* 63, *L. rhamnosus* 91 and *L. paracasei* 3 (isolated from traditional fermented milk). Group III is composed just of *L. paracasei* 137 with a similarity of 85.9% (isolated from traditional fresh cheese).

According to M. Oneca, et al. [26], the origin of the diversity of strains that colonize cheeses does not come mainly from source milk (used as a raw material for cheese making) but from other sources after milking: such as transportation systems, the environment, and equipment used to make cheese. M. Oneca, et al. [26] also adds that another possibility exists: that the strains are actually present in milk at such low levels that they are undetectable and that they then multiply and become detectable with cheese maturation (made from the same milk). This same finding of increased numbers and significant involvement by these microorganisms in cheese ripening and, more specifically, in proteolysis has been reported by Demarigny, et al. [27] in a variety of cheeses. All this explain the diversity found in our study for *lactobacillus* strains.

Biotechnological properties of LAB

Antimicrobial activity: Inhibition of the pathogenic bacteria growth is listed one of the major desirable probiotic bacteria properties. Probiotics antagonizing pathogens through production of antimicrobial compounds such as nisin bacteriocin [28], competing for pathogen binding and receptor sites as well as for available nutrients and growth factors [29,30]. Under our experiment conditions *Lactobacillus* strains -selected based on their biotechnological properties and safety profile- showed that *Lactobacillus plantarum* strains, especially *L. plantarum* 12, 21, and 63 had shown a maximum inhibition (14 mm) against *E. coli* ATCC 25922 (10^6 cfu mL^{-1}) (with no significant difference between them $P > 0.05$) (Table 3). Moreover, *Lactobacillus casei* 7a showed a good antibacterial activity (12 mm) against: *Enterococcus fecalis* ATTC 19433 (10^6 cfu mL^{-1}), *Staphylococcus aureus* ATTC 25923 (10^6 cfu mL^{-1}), and *Listeria monocytogenes* ATCC 19144 (10^6 cfu mL^{-1}) (Table 3) ($P< 0.05$). Also, *L. rhamnosus* 91 showed good antimicrobial activity against both *Enterococcus fecalis ATCC 19433* and *S. aureus* ATTC 25923 (12 mm) (10^6 cfu mL^{-1}) ($P< 0.05$). *L. plantarum* 108 showed also inhibitory activity (12 mm) against *Salmonella typhimurium ATCC 14028 (10^6 cfu mL-1) ($P< 0.05$)*, (with a significant difference with other *Lactobacillus* bacteria).

The best antibacterial profile remains that of *L. plantarum* against *E. coli*. Same results found by M.B. Pisano. et al. [31], that according to the *lactobacillus plantarum* strains tested showed an inhibitory activity toward *E. coli*, most likely due to the production of organic acids.

Proteolytic and lipolytic activity

About 95% of *Lactobacillus* spp. showed proteolytic activity by the appearance of a clear halo around the disks on a milk-based medium, and 71% showed a lipolytic activity observed by a halo

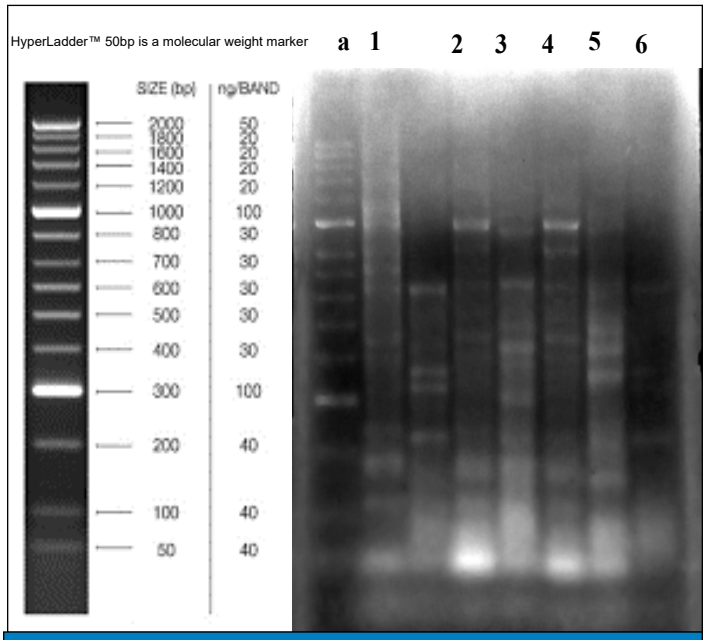

Figure 4: Agarose gel electrophoresis of rep-PCR products (for LAB isolated from fermented milk "Lben"). Lane (a) DNA marker (Hyperladder™ 50bp, Bioline) with 50 bp to 2000 bp and 300 bp, 1000 bp and 2000 bp high intensity reference bands; Lane 1, L. curvatus 81, lane 2, L. curvatus 134, lane 3, L. rhamnosus 91, lane 4, L. plantarum 108, lane 5, L. plantarum 62, lane 6, L. plantarum 57; lane 7, L. plantarum 63.

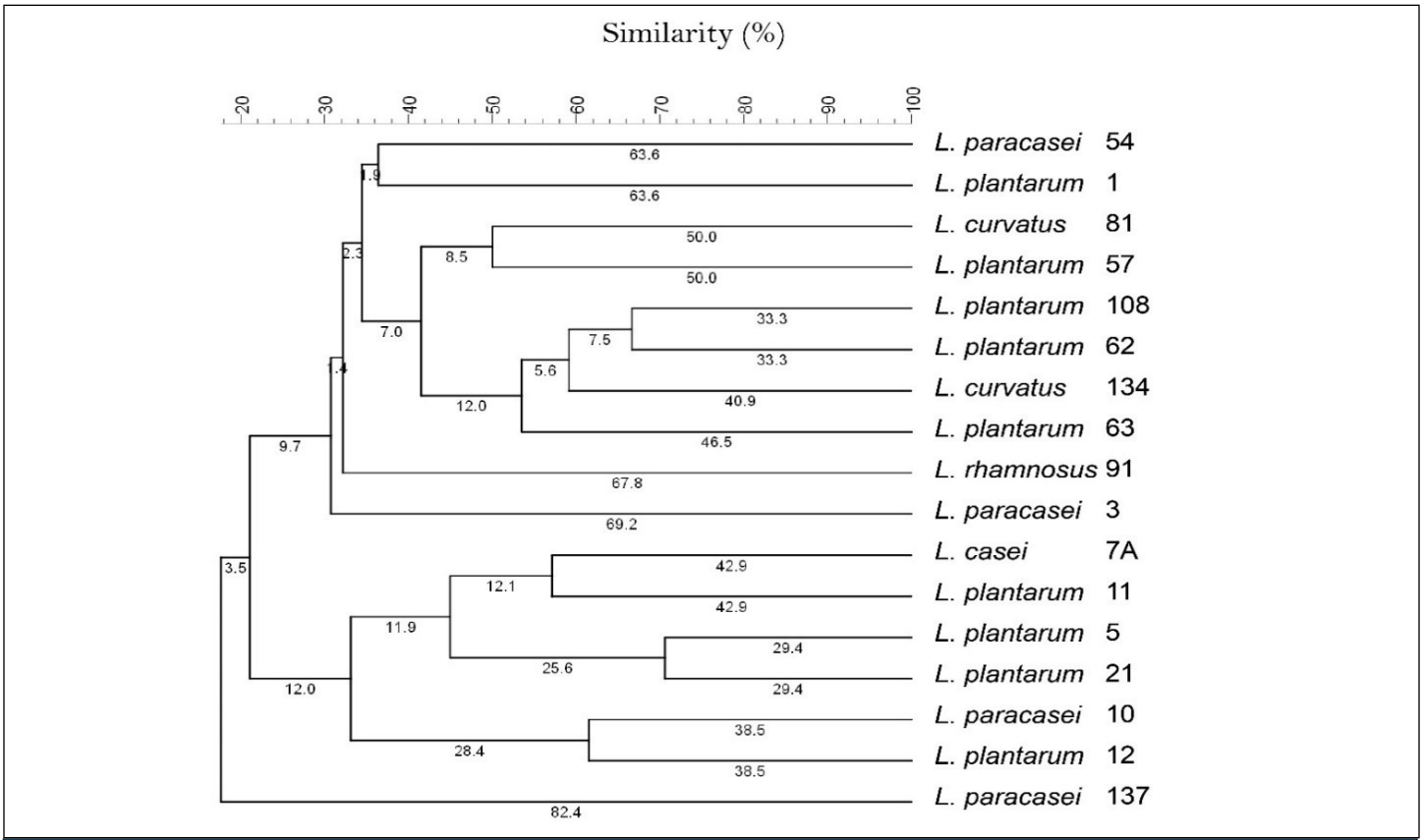

Figure 5: The UPGMA dendrogram of Lactobacillus strains was generated using the digitised images were normalised and subsequently analysed using the Bionumerics (version 7.6). The Cluster analysis of ERIC-1 and ERIC2 -PCR fingerprints of 17 Lactobacillus strains: *L. plantarum* **1,** *L. plantarum* **5,** *L. casei* **7a,** *L. paracasei 10, L. plantarum 11, L. plantarum 12, L. plantarum 21, L. curvatus 81, L. curvatus 134, L. rhamnosus 91, L. plantarum 108, L. plantarum 62, L. plantarum 57; L. plantarum 63. L. paracasei 3, voie 2, L. paracasei 54, L. paracasei 137.*

opaque around the colony.

Acidification and coagulation properties

It was found that the acid production differed with the bacterial species. About 65% of LAB strains showed strong acidification properties with a maximum acidity rate (after 24 h) of 85°D observed for *L. plantarum* (Figure 6). The coagulation time ranged from 18 h to 30 h at 37°C. Several strains of *L. plantarum* showed the fastest coagulation time ranging from 18 - 24 h.

Survival capacity: Bile salts and Acid tolerance: A set of 18 *Lactobacillus* strains selected based on their biotechnological properties and antimicrobial activity were tested for bile salts tolerance (1, 2, 3, and 4%). The results showed that all the *Lactobacillus* strains could tolerate up to 4% of the bile salts, higher than the normal intestinal concentration (2%) (Table 4).

Different viability rates were observed at a low pH of 1.5 at t = 0 min. The maximum capacity to survive was observed for the 10–5 dilution for *Lactobacillus plantarum* 1 (104.57%) (P< 0.05), followed by *Lactobacillus paracasei* 3 (102.83%) and *Lactobacillus rhamnosus* 91 (102.70%), respectively. The maximum capacity to survive at pH 2 at t = 0 min for the same 10–5 dilution was observed for *L. plantarum* 62 (104.57%) compared to other probiotics bacteria (P< 0.05), followed by *L. plantarum* 108 and *L. rhamnosus* 91 (100.00%). On the other hand, *L. plantarum* 1 also showed a maximum survival rate (103.80%) at pH = 2 at t = 30 min compared to other bacteria except for *L. plantarum* 5 followed by *L. plantarum 96* (102.41%) (P< 0.05) (Table 4). All the results were similar to their found by amaly, et al. [32], which shows

Table 4: Acid and bile salts tolerance of LAB strains

Strains	Survival in the presence of the bile salts				Survival at different pH (%SD) (D^{-5})			
					1,5		2	
	1%	2%	3%	4%	t=0	t=30min	t=0	t=30min
L. plantarum 1	(+)	(+)	(+)	(+)	104.57 ± 0.77^{d}	0	87.41 ± 3.22^{a}	103.80 ± 3.76^{de}
L. pracasei 3	(+)	(+)	(+)	(+)	102.83 ± 4.24^{cd}	0	98.39 ± 1.19^{bcd}	101.28 ± 5.17^{de}
L. plantarum 5	(+)	(+)	(+)	(+)	96.73 ± 3.72^{ab}	0	94.81 ± 10.00^{a}	83.06 ± 0.00^{a}
L. paracasei 10	(+)	(+)	(+)	(+)	91.51 ± 1.49^{a}	0	91.43 ± 5.16^{ab}	92.13 ± 4.98^{ab}
L. plantarum 12	(+)	(+)	(+)	(+)	$93.51\pm1.69^{a*}$	0	97.58 ± 0.00^{a}	94.13 ± 4.98^{ab}
L. plantarum 21	(+)	(+)	(+)	(+)	$94.63\pm1.39^{a*}$	0	95.18 ± 0.55^{a}	95.10 ± 3.17^{ab}
L. paracasei 54	(+)	(+)	(+)	(+)	$93.21\pm4.50^{a*}$	0	91.13 ± 5.16^{ab}	94.30 ± 4.84^{bcd}
L. plantarum 57	(+)	(+)	(+)	(+)	100.00 ± 2.86^{bcd}	0	95.89 ± 1.16^{a}	99.81 ± 4.77^{de}
L. plantarum 63	(+)	(+)	(+)	(+)	98.64 ± 3.28^{bc}	0	104.57 ± 1.01^{d}	94.71 ± 0.00^{bcde}
L. rhamnosus 91	(+)	(+)	(+)	(+)	102.70 ± 2.51^{cd}	0	100.00 ± 4.14^{cd}	91.06 ± 5.81^{bc}
L. plantarum 96	(+)	(+)	(+)	(+)	$98.42\pm0.00^{a*}$	0	96.04±4.64a	102.41 ± 2.23^{de}
L. plantarum 108	(+)	(+)	(+)	(+)	99.92 ± 1.98^{bcd}	0	100.00 ± 4.14^{cd}	87.18 ± 0.00^{ab}
L. curvatus 134	(+)	(+)	(+)	(+)	96.01 ± 4.47^{ab}	0	96.68 ± 2.74^{abcd}	100.00 ± 5.66^{cde}

*a-e Values with different letters within a column indicate significant differences between LAB strains ($p < 0.05$). Values are expressed as the mean ± SD of three independent experiments ($n = 3$). *Means in the same row with different superscript letters differ significantly ($P < 0.05$). D: Dilution*

that *Lactobacillus* strains isolated from dairy Moroccan products shows a good survival at low pH of acid gastric conditions (pH 2.0 and pH 3.0), and good tolerance to bile salts. Also, according to Hoque, et al. [33], *Lactobacillus* spp. Isolated from selective regional yoghurts of Bangladesh did show good survival abilities in acidic (pH 2.5) and alkaline (pH 8.5) conditions, and also with resistance to bile acid (0.05 – 0.3%) This finding revealed that the isolated *Lactobacilli spp*. could survive in extreme acidic as well as alkaline conditions.

Security aspect

None of the strains in the present study showed positive results for the safety aspects tested, All the Lactobacillus strains considered in the present study showed no DNase, or gelatinase activities.

Aggregation properties

Auto-aggregation: The auto-aggregation of *Lactobacilli* (Table 5) showed that the strains exhibited different auto-aggregation capacities, 41.19% of auto-aggregation capacity for *L. casei* 7a, and 41.21% of auto-aggregation capacity for *L. plantarum* 108 compared to other *Lactobacillus* strains (P< 0.05). Similar results were found by Gandomi, et al. According to All the *L. plantarum* strains studied showed auto-aggregation up to 74%. However, Peres, et al. [34] find that *L. plantarum*, and *L. paraplantarum* showed lower auto-aggregation capacities of 4 – 12% and according to their study the auto-aggregation of *lactobacillus* is strain-specific.

Co-aggregation: The results of the co-aggregation of *Lactobacillus* strains with pathogenic bacteria varied with the species (Table 4). *L. plantarum* (strains, 12 and 21) showed a high co-aggregation capacity (99% and 100%) against *E. coli* CCUG 47553. In contrast, *Lactobacillus paracasei* 3, followed by *L. plantarum* (53 and 57), showed a high level of co-aggregation of 44.62% and 50%, against *Listeria monocytogenes ATCC 19144*, respectively compared to other *Lactobacillus* strains (P< 0.05). Our study revealed that co-aggregation of the potential probiotic strains with bacterial pathogens was variable and depended on the species or strains, which is concordant with previous studies of Gandomi, et al. which reported that *L. plantarum* F2 showed maximum adherence to *E.*

Table 5: The aggregation properties of Lactobacillus strains (n=17)

Strains	Co-aggregation (% SD)		Auto-aggregation (% SD)	**Biofilm formation capacity
	Listeria monocytogenes ATCC 19144	Escherichia coli CCUG 47553		
L Casei 7a	0	12.09±5.65^{a*}	41.19±4.27^{ef*}	0.23±0.40^{ab*}
L paracasei 54	9.15±0.00^{a*}	6.52±3.21^{ab}	37.33±11.17^{e}	0.47±0.23^{abc}
L plantarum 1	5.06±0.00^{a}	4.90±6.93^{ab}	9.51±1.28^{a}	0.36±0.19^{ab}
L paracasei 3	50.00±0.00^{bc}	3.69±0.80^{a}	16.24±15.18^{bc}	0.43±0.22^{abc}
L plantarum 5	4.05±2.68^{a}	7.16±2.12^{a}	21.79±9.87^{bcd}	0.58±0.27^{bc}
L plantarum 12	3.49±0.00^{a}	100.00±0.00^{c}	11.10±4.61^{ab}	0.48±0.30^{abc}
L plantarum 21	14.94±12.40^{ab}	99.87±0.00^{c}	13.41±5.93^{abc}	0.14±0.00^{a}
L plantarum 57	44.62±3.83^{bc}	41.32±51.05^{ab}	12.35±2.54^{bc}	0.45±0.25^{abc}
L plantarum 62	5.92±1.50^{a}	4.27±1.48^{a}	31.42±15.56^{de}	0.72±0.10^{c}
L plantarum 63	4.30±10.35^{a}	32.07±2.51^{ab}	23.27±10.24^{cd}	0.54±0.28^{a}
L curvatus 81	19.47±10.73^{ab}	27.45±8,32^{ab}	26.30±11.74^{cde}	0.36±0.20^{ab}
L rhamnosus 91	41.18±21.78^{bc}	34.74±8.36^{a}	20.11±2.87^{bcd}	0.74±0.31^{c}
L plantarum 108	16.35±0.00^{ab}	19.82±5.31^{a}	41.21±11.57^{ef}	0.06±0.00^{a}
L curvatus 134	11.56±0.63^{ab}	21.02±18.77^{ab}	11.10±1.04^{ab}	0.35±0.22
L paracasei 137	43.89±3.59^{bc}	6.07±2.30^{a}	24.00±4.42^{de}	0.08±0.03^{a}
L plantarum 53	45.89±1.60^{bc}	17.07±2.31^{a}	13.22±1.05^{ab}	0.09±0.00^{a}
L paracasei 10	43.18±3.63^{bc}	3.07±0.80^{a}	11.82±1.06^{ab}	0.08±0.20^{a}

*a-f Values with different letters within a column indicate significant differences between LAB strains (P < 0.05). *Means in the same row with different superscript letters differ significantly (P < 0.05).Values are expressed as the mean ± SD of three independent experiments (n = 3).*

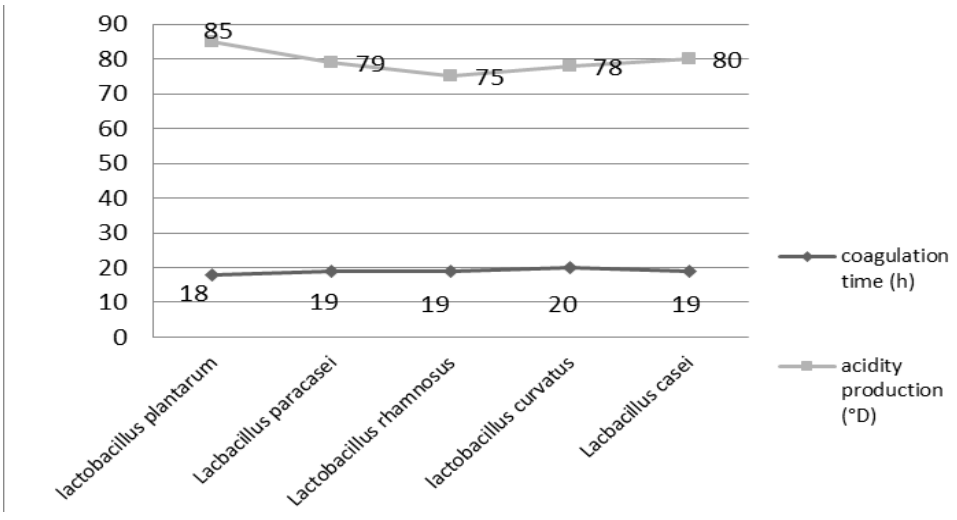

Figure 6: Acidification and clotting time of lactobacillus strains.

coli and *L. monocytogenes* with 29.6 and 29.3% co-aggregation, respectively. Moreover, and the results showed that each isolate exhibited varied co-aggregation levels with different bacterial pathogens.

Biofilm formation: Biofilms are microorganisms embedded in a matrix of extracellular polymeric substances attached to a surface [35]. Based on our results only two *Lactobacillus* strains, *L. plantarum* 62 and *L. rhamnosus* 91, were found to have the capacity to form the biofilm at a level of 0.72 and 0.74, respectively, compared to other *Lactobacillus* strains ($P< 0.05$) (Table 5). These important observed results are in agreement with those reported by Fernández, et al. [36], Metselaar, et al. [37], and van der Veen and Abee [38], according to them *L. plantarum* was found to form submerged biofilms, both as single and multi-species biofilms). On the other hand, the effect of capsular polysaccharides (cps) deletion in *L. plantarum* strains was found to be strain-dependent, with no apparent relation between hydrophobicity and adhesion [39].

While according to the study of Terraf, et al. [40] which evaluated the kinetics of biofilm formation and the chemical nature of the biofilm matrix formed by vaginal *Lactobacillus*, the biofilm matrix of *L. rhamnosus* CRL 1332 was composed of carbohydrates and proteins. Moreover, the study by Fernández, et al. [41] provided new insights into biofilm development by *L. plantarum* WCFS1 through the comparative analysis of the wild type and selected mutants deficient in the production of capsular polysaccharides, establishing a role of extracellular DNA (eDNA) in the biofilm matrix. According to the study, the deletion of Sortase A (srtA) resulted in the severe impairment of subsequent biofilm development by *L. plantarum*. In this context, the surface adhesion and biofilm formation studies revealed that none of the imposed cell surface modifications affects the initial attachment of cells to polystyrene. In contrast, biofilm formation based on crystal Violet (CV) staining was severely reduced in the ΔsrtA mutant and significantly increased in mutants lacking the cps1 cluster, compared to the wild-type. Fluorescence microscopy analysis of biofilm samples showed a higher presence of eDNA in cps1 mutants, corresponding with the increased autolysis activity. In addition, sortase A dependent proteins in biofilm formation was identified supporting cell-cell interactions as hypothesized in previous work that showed biofilm formation sensitivity to proteinase K treatment [41].

Conclusion

Moroccans' traditional dairy products: fresh cheese "Jben" and fermented milk "Lben" demonstrate that they can be the source of potential probiotic strains with a good level of genetic diversity and high percentage of similarity.

Overall, seventeen LAB isolated from local dairy products: fresh cheese, and fermented milk were demonstrated to be innocuous based on our assays. According to our result *lactobacillus* strains that demonstrated a good probiotic potential by inhibiting pathogenic bacteria, strengthening epithelial function (through their aggregation properties, and biliary salt tolerance) were: *L. plantarum* (12 and 21) isolated from fresh cheese "Jben", *L. plantarum* (63 and 108) and *L. rhamnosus* 91 isolated from fermented milk "Lben".

Our study will help advance our understanding of the contribution of the intestinal microbiota to human health and the correlation between dysbiosis and disease, including chronic intestinal diseases such as inflammatory bowel disease (IBD). This led us to conduct more probiotic mechanistic investigations into intestinal homeostasis and see potential clinical applications. Hence the importance of studying traditional dairy products as a source of new probiotics.

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