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Probiotic Potential of *Lactobacillus* strains Isolated from Known Popular Traditional Moroccan Dairy Products

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Research Article

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ABSTRACT

Eighteen *Lactobacillus* strains were isolated from Moroccan traditional dairy products and evaluated for their *in vitro* probiotic potential. The results showed that all strains tested tolerate acid gastric conditions (pH 2.0 and pH 3.0), while ten of them were bile resistant. Although no bacteriocin activity was detected *in vitro* assay for the ten bile resistant strains, but they showed strong antagonistic activity versus seven known food-borne pathogens bacteria. It was noted that none was haemolytic. In another hand, all these studied strains were found sensitive to kanamycin and tetracycline, and the majority of them were observed resistant only to one or two out of the antibiotics tested. Finally, four *Lactobacilli* strains (*Lactobacillus plantarum* LPL2, *Lactobacillus paracasei* LPAR2, *Lactobacillus paracasei* LPAR9 and *Lactobacillus brevis* LBR) were found sensitive to all antibiotics tried, and showed good hydrophobicity and adherence properties, so they could be exploited for food manufacture and scientific knowledge ends.

Keywords: Traditional dairy products; Lactobacillus; probiotics; hydrophobicity; adherence; antibiotic resistance;

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1. INTRODUCTION

Probiotics are defined as “living micro-organisms which, upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition” (Guarner et al., 1998). Most probiotic microorganisms belong to the lactic acid bacteria (LAB) group, such as *Lactobacillus* spp., and *Enterococcus* spp., or to the genus *Bifidobacterium* (Klein et al., 1998). Different bacterial species belonging to the *Lactobacillus* genus are part of the human and animal commensal intestinal flora (Vaughan et al., 2005; Zoetendal et al., 2006). It’s reported that, Lactobacilli isolated from dairy products have shown a long history of safe use (WHO/FAO, 2001). They are used widely as starter cultures in the food industry, e.g. fermented milk or meat products, alcoholic beverages, sourdough and silage (Carr et al., 2002). Furthermore, cultures of various *Lactobacillus* strains have been developed for commercial use as probiotic bacteria (e.g., Danisco (Madison WI); Probiomics (Eveleigh, Australia); Yakult (Tokyo, Japan); Danone (Paris, France); Probi AB (Lund, Sweden), etc.

Actually, a large population of probiotic bacteria is needed to carry out their benefic effect and to repel the harmful microorganisms causing disease. Indeed, some probiotic strains are rapidly killed by acid and bile, releasing active intracellular components (bacterial formylated peptides, peptidoglycan cell wall constituents, nucleotides) (Ouwehand and Salminen, 1998; Salminen et al., 1999; De Vrese et al., 2001). They may mediate a variety of health effects through numerous proposed mechanisms: inhibition of undesirable bacteria (El-Nagger, 2004; Karska-Wysockib et al., 2010), neutralization of toxins, increase of the immune response (Medici et al., 2004; Ghafoor et al., 2005), antimutagenic and anticarcinogenic activites (Boutron-Ruault, 2007; Liong, 2008; Davis and Milner, 2009; Baldwin et al., 2010), reduction of cholesterol levels (Tamaï et al., 1996; Zhang et al., 2008), control of diarrhea (Dylewski et al., 2010; Gao et al., 2010), alleviation of lactose intolerance (Guarner et al., 2005), inflammatory bowel diseases (Kruis et al., 2004; Matthes et al., 2010). They are also a source of vitamins, especially of the B group (Kneifel et al., 1992; Crittenden et al., 2003).

In order to carry out their benefic effects, probiotic strains must survive passage through the upper gastrointestinal tract (GIT), by tolerating gastric acidity and bile toxicity (Del Piano et al., 2006), and colonizing the GIT by adhering to mucin or intestinal-derived epithelial cells (Dunne et al., 2001). Moreover, probiotic strains antibiotic susceptibility should be investigated to assess their safety before their use as food additives (Parvez et al., 2006).

Currently, researchers are interested in developing efficient techniques for screening and selecting probiotics bacteria, but many challenges remain. In the present study, appropriate strategies were used for the characterization of potential probiotic *Lactobacillus* strains isolated from Moroccan fermented milk products, in order to evaluate their suitability for the addition in popular traditional fermented milk products, and/ or to develop new dairy/non-dairy probiotic foods.

2. MATERIALS AND METHODS

2.1 Bacterial Strains and Isolation

Three typically important and known Moroccan fermented dairy products, namely Jben, Semen, and Raib were used as the bacteria sources. A number of bacterial strains were isolated from such food origins using the dilution agar method. Briefly, 10 grams of each sample were weighed aseptically and homogenized in 90 ml of sterile quarter-strength

Ringer's solution in a Stomacher (Lab-Blender 400, London, England). Then, sequential decimal dilutions of the homogenate were obtained. A volume of 0.1 ml of the dilutions was plated on MRS agar medium (Fluka, Sigma-Aldrich) (de Man et al., 1960) and incubated in anaerobic conditions at 37°C for 48 h. Isolated colonies were taken randomly to be purified. The obtained purified colonies were tested for lactobacilli by microscopic examination using Gram stain and catalase production techniques. The Gram positive, catalase-negative rods were selected and stored at -20°C in MRS broth (Fluka, Sigma-Aldrich), supplemented with 15% (v/v) glycerol for further studies.

2.2 *Lactobacilli* Identification

Selected lactobacilli isolates were cultivated in MRS broth and incubated under anaerobic conditions for 24 h at 37°C. The isolates were identified at species and strain level by rep-PCR using the (GTG)5 primer (Gevers et al., 2001). Fingerprints were analyzed by the BioNumerics® V 4.5 software package (Applied Maths, Gent, Belgium) and dendrograms were obtained by UPGMA (Vauterin and Vauterin, 1992). The similarity between profiles was calculated using the Pearson correlation coefficient. To set the similarity thresholds in order to discriminate different species and strains, different cultures of control strains of Lactobacillus obtained from the Deutsche Sammlung von Microrganismen und Zellkulturen GmbH (Braunschweig, Germany) were amplified on independent occasions. Rep-PCR profiles of *Lactobacilli* isolated in this study were compared versus those included in a database holding more than 1000 identified profiles of strains of *Lactobacillus*. Isolates having profiles with a similarity index ≥ 60% were considered belonging to the same species. Thusly, to discriminate between different strains thresholds of 93 and 94% were used for *L. paracasei* and *L. plantarum*, respectively.

2.3 Tolerance to Simulated Gastric Juice

The tolerance of the strains to simulated gastric juices was tested as described by Charteris et al. (1998) with slight modifications. Stationary-phase-grown cells were harvested by centrifugation at 6,000 g for 20 min at 4°C, washed twice with 50 mmol/l phosphate buffer (pH 6.5) and suspended in 3 ml of the same buffer. Then, 1 ml of washed cell suspension was harvested by centrifugation at 12,000 g for 5 min under 4°C and resuspended in 10 ml of simulated gastric solution contained NaCl (125 mmol/l), KCl (7 mmol/l), NaHCO3 (45 mmol/l), and pepsin (3 g/l) (Fluka, Sigma-Aldrich). Final pH was adjusted to 2.0 and 3.0 using 1 mol/l HCl solution. Total viable counts were determined before and after 3 h incubation period at pH 2.0 and 3.0, at 37°C under anaerobic conditions.

2.4 Ox-Bile Cells Resistance Bacteria

The resistance of the strains to bile was performed according to Vinderola and Reinheimer, (2003). Each strain (0.2 ml of 10^7–10^8 CFU/ml concentrated culture) was inoculated into 10 ml of MRS broth containing 0.2%, 0.3%, 0.5% and 1% (w/v) Ox-bile (Biochemika, Fluka; Sigma–Aldrich) (MRS+bile). After 24 h incubation at 37°C, the optical density (OD) at 560 nm was measured and compared to a control culture (without Ox- bile). The results were expressed as the percentage of bile salts resistance compared to the control.

So percentage of resistance = (Increment of OD in MRS broth with Ox-bile / increment of OD in MRS broth without Ox-bile) × 100. Strains showing resistance percentage at the value of 0.3% of Ox-bile more than 50% were considered as bile resistance strains; and strains

showing tolerance to gastric juice and bile resistance were further tested for their antagonistic activity, antimicrobial susceptibility, haemolytic activity, surface hydrophobicity and adherence to epithelial cells.

2.5 Antibacterial Activity Determination

To carry out the antibacterial activity, the strains were tested for their inhibitory potential against some gastro-intestinal pathogens organisms with the well diffusion assay using cell or culture supernatants (Du Toi et al., 1998). Fresh overnight lactobacilli MRS cultures were harvested by centrifugation at 8,000g for 10 min, and cell-free supernatants were used directly or after were filter-sterilized (0.22 µm pore size; Serva, Heidelberg, Germany), neutralized with 1 mol/l NaOH (pH 6.5-7) and treated with catalase (0.5 mg/ml) (Sigma-Aldrich). And, the Lactobacillus cells were digested with simulated gastric juice and intestinal fluid (0.1% (w/v) pancreatin and 0.15% (w/v) Ox-bile) to simulate passage conditions through the stomach and the small intestine. Then, the digested strains, cell-free supernatants and neutralized cell-free supernatants were studied for their antimicrobial activity (Zárate et al., 2000).

To perform the antimicrobial activity, the pathogenic bacteria used as indicators included Gram-negative and Gram-positive reference strains, such as *Escherichia coli* LMHAE-SA EC 108, *Staphylococcus aureus* LMHAE-SA 105, and *Listeria innocua* LMHAE-LI 107, *Escherichia coli* ATCC25922, *Enterococcus faecalis* ATCC 25212, *Klebsiella pneumonia* CIP 53153 and Streptococcus group D. These bacteria strains were kindly provided from the Pasteur Institute (Casablanca-Morocco) and from the Laboratory of Pharmacology and Toxicology, Faculty of Medicine and Pharmacy (Rabat-Morocco). To monitor this experiment, indicator strains were cultured overnight in LB broth pH7.0 (Sigma- Aldrich) and diluted with sterile phosphate buffer saline (PBS) (pH7.2). After dilution, cells of indicator strains were mixed with 5 ml of LB soft agar (0.7% (w/v)) to reach a final concentration of 10^4 CFU/ml, and then media were poured into pre-prepared plates with 10 ml of base agar containing 2% (w/v) agar. Then, 5 mm diameter wells were cut into the agar plates and 50 µl of the lactobacilli cells (10^8 CFU/ml of physiological solution) or cell-free supernatant were placed in each well. The plates were then stored at 4°C for 4h to permit radial diffusion of the eventual antimicrobial compound. Following the anaerobic incubation at 37°C during 24 h, the plates were checked for the appearance of clear inhibition zones. Each assay was performed in triplicate.

2.6 Antimicrobial Susceptibility Tests

The minimum inhibitory concentrations (MICs) of six antibiotics were determined using the broth microdilution methods. The antibiotics and their corresponding concentration ranges were as follow: ampicillin (0–16 µg/ml), gentamicin (0–64 µg/ml), kanamycin (0–128 µg/ml), streptomycin (0–128 µg/ml) (tested against LBR only), tetracycline (0–64 µg/ml) and chloramphenicol (0–16 µg/ml). Tests were performed according to the method described by Klare et al. (2005). Briefly, overnight colonies of each studied strain were suspended in the LSM broth (LSM consists of a mixture of Iso-Sensitest broth medium (Oxoid Ltd.) (90%) and MRS broth medium (10%) adjusted to pH 6.7) to a density of approximately OD_{560} 0.4. Cell suspension was diluted 1:1000 in LSM broth and 100 µl of the obtained suspension were inoculated into each well of the microtiter plates (Iwaki brand, Scitech div. Asahi Techno Glass, Japan) containing diluted antibiotics in LSM broth and incubated anaerobically at 28°C for 48 h. To perform the analysis, tests were conducted in duplicate for each bacteria

strain. And, MICs were defined as the lowest antibiotic concentration causing no organism growth. Finally, the results of susceptibility status was interpreted according to the recent European Food Safety Authority (EFSA) document on the update of the criteria used in the assessment of antibiotics bacterial resistance of human or veterinary importance (EFSA, 2008).

2.7 Haemolytic Activity

To determine bacteria haemolytic activity, blood haemolysis was evaluated on Columbia agar plates (Oxoid) supplemented with 5% sheep blood. Each bacterial suspension was streaked in the blood agar plates. After 24h incubation at 37°C, the plates were examined for signs of β-haemolysis (clear zones around colonies), α-haemolysis (a green-hued zone around colonies) or γ-haemolysis (no halo around colonies) (Maragkoudakis et al., 2009).

2.8 Cells Surface Hydrophobicity Assay

Bacterial adhesion to hydrocarbons tested, was determined and results were expressed according to the method described by Perez et al. (1998), modified as follows. Strains were grown in MRS broth at 37°C for 18h and centrifuged at 12,000 g for 5 min at 4°C. The obtained pellet was washed twice in 50 mmol/l phosphate buffer saline pH 6.5, and resuspended in the same buffer. Then, one milliliter of the suspension was taken and the absorbance at 560 nm was measured. Three ml of the bacterial suspension was mixed with 0.6 ml of n-hexadecane (Sigma-Aldrich) and stirred for 120s using a vortex instrument. Then, the phases were allowed to separate for 1h at room temperature. And, the aqueous phase was carefully removed and the absorbance was measured at 560 nm. Thusly, the hydrophobicity percentage was expressed as follows: hydrophobicity % = $[(A_0 – A)/A_0] \times 100$, where A_0 and A are the absorbance values of the aqueous phase before and after contact with n-hexadecane, respectively. The hydrophobicity assay determinations were performed in triplicate.

2.9 *In Vitro* Lab Epithelial Cells Adherence Test

The method described by Annika et al. (1983) was used for the preparation of epithelial cells. Segment of the rat ileum was opened and washed with sterilized PBS (0.1mol/l, pH 7.2). It was held in PBS at 4°C for 30 min to remove the surface mucus, and then washed three times with PBS. Epithelial cells were scrapped into sterilized PBS. The cell suspension obtained was examined by microscopy to ensure that contaminated bacteria had been removed and the final epithelial cell concentration was adjusted to approximately 5×10^4 cells/ml. Then, the adherence assay was studied according to the method reported by Pedersen and Tannock, (1989) and Lin et al. (2007). The selected strains showing an adhesion efficiency of at least 15 bacteria per epithelial cell were considered positive and 10 epithelial cells were examined per analysis.

3. RESULTS

3.1 Bacterial Strains, Isolation and Identification

From three types of the fermented dairy products, thirty nine strains were determined Gram positive rod shaped, non-spore forming, and catalase negative which indicated the typical basic characteristics of lactobacilli. Among these 39 strains studied, 13, 5 and 21 strains

were, respectively, from Jben, Smen, and Raib. Relatively to the rep-PCR using the (GTG)5 primer, the results suggested that all selected bacteria isolates (39) were identical to three *Lactobacillus* species and a total of 18 strains were identified as: *Lactobacillus brevis* (one strain), *Lactobacillus plantarum* (5 strains) and *Lactobacillus paracasei* (12 strains) (Table 1).

Table 1. Species and corresponding origin of *Lactobacillus* strains

Strain	Code	Origin
Lactobacillus plantarum 1	LPL1	Jben: soft white cheese (traditional product)
Lactobacillus plantarum 2	LPL2	Jben : soft white cheese (traditional product)
Lactobacillus plantarum 3	LPL3	Smen : fermented butter (traditional product)
Lactobacillus plantarum 4	LPL4	Smen : fermented butter (traditional product)
Lactobacillus plantarum 5	LPL5	Smen : fermented butter (traditional product)
Lactobacillus paracasei 1	LPAR1	Jben : soft white cheese (traditional product)
Lactobacillus paracasei 2	LPAR2	Riab: cow curd milk (traditional product)
Lactobacillus paracasei 3	LPAR3	Jben : soft white cheese (traditional product)
Lactobacillus paracasei 4	LPAR4	Riab: cow curd milk (traditional product)
Lactobacillus paracasei 5	LPAR5	Jben : soft white cheese (traditional product)
Lactobacillus paracasei 6	LPAR6	Jben : soft white cheese (traditional product)
Lactobacillus paracasei 7	LPAR7	Jben : soft white cheese (traditional product)
Lactobacillus paracasei 8	LPAR8	Riab: cow curd milk (traditional product)
Lactobacillus paracasei 9	LPAR9	Jben : soft white cheese (traditional product)
Lactobacillus paracasei 10	LPAR10	Jben : soft white cheese (traditional product)
Lactobacillus paracasei 11	LPAR11	Jben : soft white cheese (traditional product)
Lactobacillus paracasei 12	LPAR12	Jben : soft white cheese (traditional product)
Lactobacillus brevis	LBR	Jben : soft white cheese (traditional product)

3.2 Tolerance to Gastric Juice and Ox- Bile Resistance Properties

In order to evaluate the ability of bacteria strains to support stomach aggressive conditions, all tested lactobacilli strains were exposed to acid and Ox-bile solutions. The results indicated that the tested strains tolerated simulated gastric juice acid conditions (Table 2). The cell viable counts decreased about 1-1.35 log CFU/ml for only three strains after 3h incubation with simulated gastric juice at the pH 2 or pH 3. The residual counts were more than 10^7 CFU/ml even after 3 h of incubation for all tested strains.

In another hand, the results obtained also showed that only ten strains were observed resistant to 0.3% of Ox-bile (percentage of resistance ≥ 50%), corresponding to survival percentages ranging from 54.05 ± 2.45 to 89.07 ± 1.26 % after 24 h incubation. The other strains such as LPL3, LPAR4 and LPAR5, were inhibited dramatically in presence of bile. And, between bile resistant strains, LPL2, LPAR1, LPAR2, LPAR9, LPAR11 and LBR bacteria were found having capability to grow also at high Ox-bile concentrations (0.5% and 1%) (Table 2).

Table 2. Effects of gastric juice at different pH and the Ox-bile on the survival of *Lactobacillus* strains

Strains of *Lactobacilli*	Viable counts (log CFU/ml)[a]			Percentage of resistance of *Lactobacillus* strains at different Ox-bile concentrations after 24 h of incubation			
	0h	3h		0.2%	0.3%	0.5%	1.0%
		pH 2.0	pH 3.0				
LPL1	9.28 ± 0.01	8.08 ± 0.14*	8.73 ± 0.37	78.82 ± 0.28	54.91 ± 0.33	42.21 ± 1.25	39.66 ± 1.56
LPL2	10.13 ± 0.01	9.20 ± 0.07*	9.37 ± 0.26*	92.31 ± 0.93	66.8 ± 0.95	61.34 ± 0.68	59.26 ± 0.70
LPL3	7.89 ± 0.02	7.63 ± 0.02*	8.15 ± 0.00*	24.96 ± 0.26	22.45 ± 2.26	15.48 ± 1.39	17.57 ± 0.70
LPL4	8.31 ± 0.02	9.19 ± 0.01*	9.08 ± 0.01*	50.33 ± 1.50	25.57 ± 1.99	17.44 ± 1.46	15.73 ± 0.58
LPL5	7.58 ± 0.09	7.43 ± 0.02	7.54 ± 0.01	34.62 ± 3.88	24.59 ± 2.83	20.16 ± 1.31	18.05 ± 0.83
LPAR1	9.67 ± 0.05	8.67 ± 0.13*	9.93 ± 0.04	84.13 ± 1.38	69.03 ± 0.43	61.05 ± 2.20	59.09 ± 1.45
LPAR2	9.55 ± 0.08	9.20 ± 0.11*	9.39 ± 0.04	87.69 ± 2.29	71.41 ± 1.16	59.00 ± 1.47	57.69 ± 1.14
LPAR3	8.15 ± 0.01	7.79 ± 0.29	7.90 ± 0.07	76.31 ± 5.90	15.00 ± 0.73	3.71 ± 0.18	3.66 ± 0.23
LPAR4	9.43 ± 0.03	9.41 ± 0.04	9.33 ± 0.04	4.47 ± 0.19	4.60 ± 0.02	4.33 ± 0.12	4.62 ± 0.28
LPAR5	9.21 ± 0.02	8.30 ± 0.06*	7.86 ± 0.01*	56.20 ± 1.71	12.24 ± 0.86	13.03 ± 2.65	10.60 ± 0.23
LPAR6	9.14 ± 0.03	9.24 ± 0.01	9.26 ± 0.05	65.79 ± 1.63	26.41 ± 0.44	18.40 ± 2.63	14.57 ± 0.70
LPAR7	9.46 ± 0.01	8.64 ± 0.05*	9.50 ± 0.08	76.69 ± 1.11	49.38 ± 0.88	44.00 ± 0.35	42.52 ± 1.21
LPAR8	9.37 ± 0.01	9.18 ± 0.08	9.56 ± 0.16	90.17 ± 1.20	65.57 ± 0.94	27.26 ± 6.71	15.26 ± 0.70
LPAR9	9.21 ± 0.10	9.10 ± 0.02	9.42 ± 0.04	88.26 ± 1.35	74.25 ± 1.72	61.24 ± 0.66	60.33 ± 0.39
LPAR10	9.80± 0.13	9.11 ± 0.13*	9.52 ± 0.24	84.80 ± 0.92	54.05 ± 2.45	37.36 ± 1.33	36.64 ± 1.05
LPAR11	9.28 ± 0.04	9.39 ± 0.17	9.39 ± 0.16	94.70 ± 0.79	69.70 ± 0.46	64.82 ± 4.02	60.55 ± 1.30
LPAR12	8.57 ± 0.07	8.43 ± 0.02	8.51 ± 0.02	56.23 ± 1.70	66.54 ± 2.17	8.58 ± 1.29	7.46 ± 1.07
LBR	10.11 ± 0.28	9.72 ± 0.18	9.63 ± 0.04*	98.75 ± 6.38	89.07 ± 1.62	72.62 ± 5.16	65.20 ± 1.56

[a] *Each value in the table represents the mean value ± standard deviation (SD) from triplicate. *Counts significantly different from those at 0 h (P<0.05).*

3.3 Antagonistic Activity of *Lactobacillus* Strains

As elucidated in Table 3, all Lactobacillus cells showed clear inhibition toward most tested indicator bacteria. Overall, the acid cell-free extracts of lactobacilli were markedly inhibitory against all potential pathogens. Of note, neutralization and/or treatment with catalase affect the antibacterial activity of all strains (no inhibition showed).

3.4 Antibiotics Susceptibility and Haemolytic Activity

In this research, the MIC distributions of lactobacilli strains relatively to six tested antibiotics are shown in Table 4. The bacteria, LPL1, LPAR1, LPAR10, LPAR11 and LPAR12, strains showed MICs higher than EFSA's breakpoints for the ampicillin antibiotic. All MICs determined for kanamycin and tetracycline were equal to or below the EFSA's breakpoint. In addition, the LPL2, LPAR2, LPAR9 and LBR strains showed MICs equal to or below the EFSA's breakpoint for all antibiotics tried. Of note, no strain, tested in this work, exhibited β-haemolytic activity when grown in Columbia blood agar. While, most strains were γ-haemolytic, and only two strains LPAR3 and LPAR12 exhibited α-haemolytic activity.

3.5 Bacteria Hydrophobicity and Adherence Properties

In order to complete probiotic criteria, the hydrophobicity and adherence properties of selected bacteria strains were performed. The calculated value for the hydrophobicity ranged from 37.80 to 85.67, 21.06 to 88.00 and 76.33 %, respectively, for *Lactobacillus paracasei*, *Lactobacillus plantarum*, and *Lactobacillus brevis* as shown in Table 5. The adherence assay illustrated all the strains tested that possessed capability to adhere on the rat ileum epithelial cells (more than 20 bacteria per epithelial cell (Figure 1).

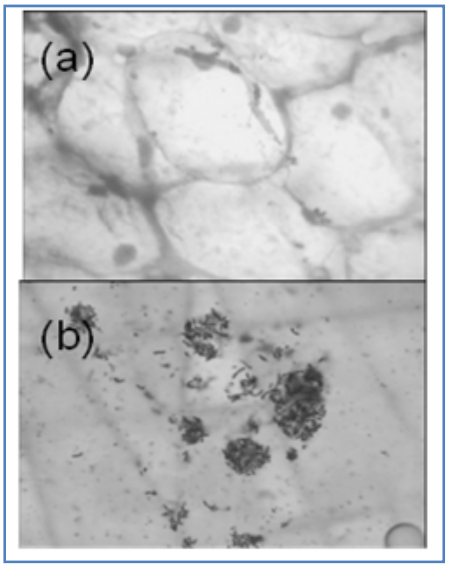

Fig. 1. Adherence of *Lactobacillus* strains to the crop epithelium from rat.
Experimental conditions were described in Materials and Methods. (1) Crop epithelium from rat only; (b) rat epithelial cells colonized (adherence phenomena) by Lactobacillus paracasei LPAR9. The magnification fold is 100X

Table 3. The antimicrobial activity of *Lactobacillus* strains against pathogens bacteria

Strains of *Lactobacillus*	Inhibition of indicator microorganisms																				
	Listeria innocua LMHAE-LI 107			*Escherichia coli* LMHAE-SA EC 108			*Staphylococcus aureus* LMHAE-SA 105			*Escherichia coli ATCC25922*			*Enterococcus faecalis* ATCC 25212			*Streptococcus D*			*Klebsiella pneumonia* CIP 53153		
	C	S	TS	C	S	TS	C	S	TS	C	S	TS	C	S	TS	C	S	TS	C	S	TS
LPL1	++	++	-	++	++	-	++	++	-	++	++	-	++	++	-	++	++	-	++	++	-
LPL2	++	++	-	++	++	-	++	++	-	++	+ +	-	++	+ +	-	++	++	-	++	++	-
LPAR1	++	+	-	++	+ +	-	++	+	-	++	+	-	++	+	-	++	+	-	++	+	-
LPAR2	++	++	-	++	++	-	++	++	-	++	++	-	++	+ +	-	++	+ +	-	++	++	-
LPAR3	++	++	-	++	++	-	++	++	-	++	++	-	++	++	-	++	++	-	++	+ +	-
LPAR9	++	++	-	++	++	-	++	++	-	++	+ +	-	++	++	-	++	+ +	-	++	++	-
LPAR10	++	++	-	++	+ +	-	++	++	-	++	+	-	++	++	-	++	++	-	++	++	-
LPAR11	++	++	-	++	+ +	-	++	++	-	++	++	-	++	++	-	++	++	-	++	++	-
LPAR12	++	++	-	++	++	-	++	++	-	++	+	-	++	++	-	++	+	-	++	+	-
LBR	++	++	-	++	+ +	-	++	++	-	++	++	-	++	+ +	-	++	++	-	++	++	-

Note: -. No inhibition; +. inhibition zone between 2 and 6 mm; ++. inhibition zone larger than 6 mm. C: Cells of Lactobacilli in fresh MRS broth; S: Cell-Free supernatant ; TS: Cell-Free supernatant adjusted to pH 6.5-7 and treated with catalase. Results are averages of three experiments.

Table 4. Antibiotics susceptibility of *Lactobacillus* strains

Lactobacillus strains	Antibiotics					
	Ampicillin	Gentamicin	Kanamycin	Streptomycin	Tetracycline	Chloramphenicol
LPL 1	R	S	S	n.d	S	S
LPL2	S	S	S	n.d	S	S
LPAR1	R	R	S	n.d	S	S
LPAR2	S	S	S	n.d	S	S
LPAR3	S	S	S	n.d	S	R
LPAR9	S	S	S	n.d	S	S
LPAR10	R	S	S	n.d	S	S
LPAR11	R	S	S	n.d	S	R
LPAR12	R	S	S	n.d	S	S
LBR	S	S	S	S	S	S

R: Resistant, S: susceptible, n.d. not determined

Table 5. Hydrophobicity percentage and adhesion efficiency of the *Lactobacillus* strains

Lactobacillus strains	Hydrophobicity[a] (%)	Adhesion efficiency
LPL1	21.06 ± 2.84	+
LPL2	88.00 ± 1.00	+
LPAR1	37.44 ± 0.31	+
LPAR2	78.33 ± 2.08	+
LPAR3	67.00 ± 1.00	+
LPAR9	85.67 ± 3.51	+
LPAR10	34.70 ± 8.30	+
LPAR11	35.44 ± 0.64	+
LPAR12	50.73 ± 5.67	+
LBR	76.33 ± 4.50	+

[a] *Experiments were performed in triplicate.*
"+" Represents the mean number of adherent bacteria on rat epithelial cells is more than 15 bacteria/epithelial cell

4. DISCUSSION

In the present research, *Lactobacilli* strains selected from typically Moroccan fermented dairy products were tested for their *in vitro* probiotic criteria. Tolerance to acidity and Ox-bile, antagonistic activity against pathogens, adherence to epithelial cells and safety represent, generally, the most important properties enabling a microorganism to be considered as an effective probiotic (Kaur et al., 2002; Stadler and Viernstein, 2003).

It has been reported previously that, the acidity has been known the most negative effect on growth and viability of lactobacilli during their passage through the stomach (Charteris et al., 1998). Because, the pH in human stomach ranges from 1.5 to 4.5 depending on the intervals of feeding or the food variability, and from the duration of food digestion which can take up to 3 h. Some authors proposed that strains intended for probiotic purposes should be screened according to their tolerance to pH 2.5 in an HCl-acidified culture medium during four hours (Pennacchia et al., 2004; Klingberg et al., 2006). In this research, the strains tested were found able to tolerate three hours of acid exposure with pH 2.0 and pH 3.0. These important observed results are in agreement with those reported by Charteris et al. (1998), Dunne et al. (2001), Fernández et al. (2003), El-Nagger, (2004), Petros et al. (2006) and Burns et al. (2008). It's also noted; that all strains studied tolerated pH 2 with the low survival rate is 89.65%, which is very interesting for the probiotic field. In another hand, these results are note in agreement with those obtained from similar previous studies, where *Lactobacillus* strains were viable even after being exposed to pH values of 2.5–4.0, but showed reduced viability at lower pH values (Mishra and Prasad, 2005; De Angelis et al., 2006; Wang et al., 2010). Although, *L. paracasei, L. plantarum* and *L. brevis* strains tested were isolated from dairy products, these species are normal inhabitants of the intestinal tract of healthy humans (Wall et al., 2007). For this reason, they could be adapted to resist the biological barriers present in the gut as gastric acidity, bile salts, lysozyme, etc.

Certain studies reported before, that bile tolerance is also considered an important factor which affects the LAB viability (Begley et al., 2005; Pacheco et al., 2010). Bile is a result from a digestive secretion that can play a capital role in the lipids emulsification and has the ability to affect the phospholipids, cell membranes proteins and disrupt cellular homeostasis (Burns et al., 2008). The relevant physiological concentrations of human bile salts range from 0.3 to 0.5% (Zavaglia et al., 1998; Dunne et al., 2001). According to Gilliland et al. (1984) and Goldin and Gorbach, (1992), the concentration 0.3% bile salts is considered as critical for resistant strains screening and the same level is critical for the human probiotics selection. Because of their similarity, a value of 0.3% Oxgall (Ox-bile) solution is the most used to substitute human bile salts (Gilliland and Walker, 1990; Noh and Gilliland, 1993; Chou and Weimer, 1999; Brashears et al., 2003). Relatively to the bile resistance phenomenon, 10 out of 18 resistant lactobacilli strains to 0.3% Ox-bile (with resistance % ≥ 50) were obtained and identified as 2 *L. plantarum*, 7 *L. paracasei* and 1 *L. brevis*.

In addition, 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 (del Miraglia and De Luca, 2004), competing for pathogen binding and receptor sites as well as for available nutrients and growth factors (Servin, 2004; Makras and De Vuyst, 2006; Parvez et al., 2006). Under our experiment conditions none of the lactobacilli strains showed bacteriocin activity against indicator organisms spectrum used, since no inhibition was observed when supernatants (pH 6.5) were tested. Therefore, it was observed that Lactobacilli cells present an efficient anti-pathogen activity indicating a fortiori that these strains could also be active in the GIT.

Safety confers another essential property of probiotic bacteria. These healthy organisms are powerful dietary supplements that help human rebuild the balance of beneficial micro organisms in their gastrointestinal tract and reverse the imbalances that may contribute to the onset of chronic conditions. In this way, the food industry will need to carefully assess the safety and efficacy of all new species and probiotic strains before their incorporation into food products (Parvez et al., 2006). According to the EFSA's guidance, micro-organism showing MICs same or less than EFSA's breakpoint for a specific antimicrobial is defined as susceptible to this antimicrobial (EFSA, 2008). In the present research, only four bacteria strains (LPL2, LPAR2, LPAR9 and LBR) are observed susceptible to all tested antibiotics. While, all lactobacilli strains are susceptible to tetracycline and kanamycin, only two strains are resistant to chloramphenicol (LPAR3 and LPAR11). Thus, many studies confirm that Lactobacilli are generally susceptible to antibiotics inhibiting the synthesis of proteins, such as chloramphenicol, erythromycin, clindamycin and tetracycline (Temmerman et al., 2002; Coppola et al., 2005; D'Aimmo et al., 2007). In general, glycopeptide, aminoglycoside and sulfamethoxazole resistance has been formerly described in LAB species (Elisha and Courvalin, 1995; Elkins and Mullis, 2004), and in all cases it has been associated with their natural and intrinsic resistance due to membrane impermeability, probably complemented by potential efflux mechanisms resistance (Elkins and Mullis, 2004). Intrinsic resistance is not horizontally transferable, and poses no risk in non-pathogenic bacteria (Mathur and Singh, 2005).

The adherence to gut is so an important criterion to select probiotic bacteria. Indeed, the probiotic ability to adhere to the intestinal epithelium is regarded as a prerequisite to colonize the human GIT for exerting beneficial effects, such as the exclusion of enteropathogenic bacteria (Collado et al., 2005; Marco et al., 2006). The ability of probiotics to adhere to epithelia is studied *in vitro* by evaluating the cells surface hydrophobicity toward n-hexadecane (Kiely and Olson, 2000; Schillinger et al., 2005). In this work, certain strains

assayed have showed a variable hydrophobicity degree, when LPL2 and LPAR9 were the highest hydrophobic strains. Thus, the ten bacteria strains tested were found able to adhere efficiently to rat epithelial cells. No relationship between hydrophobicity and bacterial adhesion was observed. Although, the strain that exhibited low degree of hydrophobicity showed good adhesion ability (more than 15 bacteria per epithelial cell). Similarly, Nishikawa et al. (1991), Ouwehand et al. (1999) and Mellor et al. (2009) did not found this correlation between cell surface hydrophobicity and adhesive ability of the strains. It's also reported that the adhesion capability depends upon the strain origins as well as their surface properties (Morata de Ambrosini et al., 1998). Furthermore, mucus adhesion properties are considered more dependent on the LAB strain than on the host (Rinkinen et al., 2003).

5. CONCLUSION

In conclusion the results obtained in this research considered as the first one in Morocco, indicated, that among 18 *Lactobacillus* strains studied, 10 strains: LPL1, LPL2, LPAR1, LPAR2, LPAR3, LPAR9, LPAR10, LPAR11, LPAR12 and LBR; showed good resistance to gastrointestinal conditions and undesirable bacteria inhibition. Concerning the safety and adherence properties, the strains LPL2, LPAR2, LPAR9 and LBR were observed susceptible to all antibiotics tested under the search conditions and were the highest hydrophobic strains. Moreover, these results offer them potentially useful as probiotics in human and/or animal foods. However, further work is needed to carry out the *in vivo* potential study of these selected strains.

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