

Characterization and Selection of Lactobacilli with Probiotic Properties from Cow's Raw Milk of "Bororo" Cattle Breeders in Dschang (Cameroon)

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Abstract: This study aimed at isolating potential probiotic lactobacilli strains from cow's raw milk collected near the small cattle breeders in Dschang (Cameroon) and applying established *in vitro* tests to screen those strains that could be used as novel probiotic strains in the food industry. Thirty four lactobacilli isolates obtained from raw cow's milk were characterized and selected according to their preliminary probiotic properties including tolerance to acid and bile salts, ability to assimilate cholesterol, sensitivity to antibiotics and antimicrobial activities. Among the several requirements, the acid tolerance as well as the bile salt tolerance was strain dependent. Nine isolates exhibited the highest surviving percentage at pH 3 and 2. Six acid tolerant isolates presented an excellent tolerance at 0.2 and 0.4 % bile salts. The acid tolerant and bile tolerant isolates were sensitive to most of the antibiotics tested. Six acid and bile tolerant isolates (6M, 11M, 12M, 21M, 48M) exhibited antimicrobial activity against *Staphylococcus aureus*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Escherichia coli*. These isolates reduced between 20.35 and 45.21 mg of cholesterol per g (dry weight) of cells *in vitro*. They were identified using API 50CH kits and repetitive genomic element-PCR (Rep-PCR) fingerprinting as *Lactobacillus plantarum*. According to the obtained results, these *Lactobacillus plantarum* strains (6M 11M, 12M, 21M and 48M) were found to be the most promising strains with preliminary probiotic properties.

Key words: Antibiotic susceptibility, cholesterol assimilation, *Lactobacillus plantarum*, probiotic, Rep-PCR

INTRODUCTION

The probiotic concept has been defined by Fuller to mean "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller, 1991; Guarner and Shaafsma, 1998). Criteria for a bacterium to be probiotic include:

- human origin
- non-pathogenic properties
- resistance to technologic processing, including viability in delivery vehicles
- stability in acid and bile
- adhesion to target epithelial tissue
- ability to persist within the gastrointestinal tract
- production of antimicrobial substances

- ability to positively influence immune function and positively influence metabolic
- activities (Du Toit *et al.*, 1998; Maragkoudakis *et al.*, 2006).

Numerous studies from subsequent research programs reported the generally unsubstantiated health-promoting properties of lactic acid bacteria (LAB) from fermented dairy products in animals and humans. In order to produce their health-promoting properties, probiotics must be carefully selected and screened. Some of the selection criteria include resistance to gastric acid and bile salts, adherence to gut epithelial tissue, susceptibility to antibiotics, ability to reduce cholesterol level in the blood (Du Toit *et al.*, 1998; Dunne and Mahony, 2001; Brashears *et al.*, 2003; Maragkoudakis *et al.*, 2006). During the last decade, the market of functional food

products containing probiotics has been undoubtedly the fastest growing area of new food product development. For continuous manufacturing of these functional foods, the food industries need new probiotic cultures with more beneficial physiological effects beyond those of good nutrition without adverse effects for innovations. This specificity generates a need to screen cultures from different origins especially in the area of the world where the wild microflora present in fermented products have not yet been characterized. Some research work has been carried out on the microflora of dairy products in Cameroon (Zambou *et al.*, 2004, 2008), but very few information on the beneficial health effects of the wild microflora found in these products are published. It is therefore important to initiate comprehensive studies to screen probiotics lactobacilli strains in cow's raw milk from this geographical region of the world.

This study therefore aimed at isolating potential probiotic lactobacilli strains from cow's raw milk collected near the small cattle breeders in Dschang (Cameroon) and applying established *in vitro* tests to screen those strains that could be used as novel probiotic strains in the food industry.

MATERIALS AND METHODS

Isolation and identification of lactobacilli strains:

Twenty samples of raw milk used in this work were collected from four Bororos cattle breeders living in Dschang area (Cameroon) from May to August 2008. Milk samples were incubated at 37°C until coagulation. A portion (1 mL) of the coagulated milk was aseptically sampled into 9 mL of sterile MRS broth (Merk) supplemented with 0.5% (W/V) of L-cysteine-HCl and homogenized. After incubation at 37°C for 24 h, the cultures were then streaked on MRS agar media supplemented with 0.5% cysteine-HCl. Cultures streaked on MRS were incubated under anaerobic conditions at 37°C for 48 h, after which, single colonies were picked off the plates and subcultured in MRS broth before microscopic examination. The cultures were examined microscopically following gram staining (Harigan and Mc Cance, 1976) and later catalase tested. They were initially selected on the basis of gram reaction, morphology and catalase activity. Purified isolates of rods were stored at -20°C in sterile reconstituted skim milk (12.5% (W/V)) supplemented with 15% (V/V) glycerol.

Identification of *Lactobacillus* strains was carried out using morphological and physiological methods (Sharpe and Wheeler, 1979). The ability of isolates to grow at 45°C and 10°C was assessed in MRS broth after 2 days and 10 days of incubation respectively. In addition, all isolates were tested for CO₂ production from glucose. All tests were carried out in triplicates. Isolates subsequently shown to express potential probiotic properties were identified using API 50CH kits (Biomerieux, Marcy

Etoile France) and repetitive genomic element-PCR (Rep-PCR) fingerprinting.

API 50CH identification was performed according to the manufacturer's instructions. Interpretations of the fermentation profiles were facilitated by systematically comparing all results obtained for the isolates studied with information from the computer-aided database API LAB Plus V3.2.2. (<http://www.biomerieux.com>)

Rep-PCR genomic fingerprinting: Total DNA was extracted from 1.6ml of fresh cultures in the exponential phase using the Wizard DNA purification Kit as described by the manufacturer (Promega). The quality of DNA obtained was estimated by comparison to known standards in ethidium bromide stained 0.8% agarose gel. The DNA concentration of each sample was adjusted to 25 ng/μL in a 25 μL PCR mixture. Amplification was performed in a 25μl reaction volume, typically containing 25 ng genomic DNA, 0.3 μM BOXAIR primer (5'-CTACGGCAAGGCGACGCTGACG-3'), pure Taq ready-To-Go PCR Beads (Amersham Biosciences, Sweden) which included deoxynucleoside triphosphate at a concentration of 200 μM, 2.5 U of puRe Taq DNA polymerase, 10 mM Tris HCl, (pH 9.0), 50 mM KCl, 15 mM MgCl₂ and enough sterile deionized water to bring the volume to 2 μL. PCR amplification was performed in a Flexigene thermal cycler (technique, UK). The PCR program described by De Urraza *et al.* (2000) was used. PCR products were separated by electrophoresis at 50V on a 2% (w/v) agarose (Amersham Biosciences, Sweden) and the DNA were detected by UV transillumination after staining with ethidium bromide (10 mg/mL). The molecular sizes of the amplified DNA fragments were estimated by comparison to a 100bp DNA ladder (Promega) and were photographed using Polaroid film. The Rep profiles were processed using the Gel Compar version 5.00 software (Applied Maths, Kortrijk, Belgium). The reference lactobacilli strains included in the rep-PCR identification process were obtained from the database of the Laboratory of Microbial Biochemistry-University of Alexandria

Inhibition of the growth of pathogenic bacteria: The LAB isolates were tested for inhibition of the growth of some pathogenic bacteria using the spot on the lawn test as described by Geis *et al.* (1983). An aliquot (2 μL) of an overnight LAB culture was spotted onto MRS agar plates and incubated anaerobically at 30°C for 48 h. The plates were subsequently overlaid with soft MRS agar (0.75% agar) or soft MHA containing 1% indicator strains respectively, and then incubated anaerobically at 30°C or aerobically at 37°C, on the basis of the tested organisms, for 24 h. Only isolates showing largest inhibition diameter zones were selected for the next steps.

Pathogenic bacteria used as indicator strains in this study including *Staphylococcus aureus* ATCC25922,

Salmonella Typhi ATCC 6539, *Pseudomonas aeruginosa* ATCC27853, *Escherichia coli* ATCC 13706, were cultured at 37°C and maintained on Mueller Hinton Agar (MHA, Conda, Madrid, Spain) slants.

Acid tolerance of lactobacilli isolates: MRS broth was adjusted to pH 2, 3 and 7 respectively, using HCl or NaOH. The MRS broth media were inoculated with 10% (v/v) of overnight culture and incubated at 37°C. Bacterial growth was monitored by determination of optical density at 620nm after 6 and 24h incubation period at 37°C. The percent difference between the variation of optical density (DO) at pH7.0 (DDO_{pH7}) and the variation of optical density (DO) at pH2 or 3 (DDO_{pH2 or 3}) would give an index of isolates surviving that can be expressed as follows:

$$\text{Surviving}(\%) = \frac{\Delta DO_{pH7} - \Delta DO_{pH2 \text{ or } 3}}{\Delta DO_{pH7}} \times 100$$

Classification criteria included four arbitrary level of acid condition tolerance: excellent if the isolate survived at pH 2 after 24 h; very good if the isolate survived at pH 2 after 6 h but not after 24 h; good if the isolate survived at pH 3 after 24 h but not at pH2; poor if the isolate did not survive in any experimental condition.

An isolate survived if it demonstrated a surviving percentage equal or greater than 50%.

Selected isolates showing a good surviving percentage at pH 2 were cultured on MRS agar medium for 24 h at 37°C. Colonies of isolates were collected and suspended in 0.1M citrate buffer pH3, and the turbidity of cells suspensions were compared to 4 Mc Farland (12X 10⁸cfu/mL) followed by serial dilution and plate counting (Verdenelli *et al.*, 2009). At the time of 0 and 5 h incubation at 37°C, each strain was cultured in MRS agar and incubated anaerobically at 37°C for 48 h. Results were expressed as the percent (log₁₀cfu) of resistant cell.

Bile salt tolerance of lactobacilli strains: A fresh culture of the selected strain was inoculated at 10% (v/v) into MRS broth containing Bile salt N.3 (Oxoid) at different concentrations (0, 0.2 and 0.4% (w/v) and incubated at 37°C. The growth of the isolates was monitored at 0, 6 and 24 h by measuring the absorbance of the culture broth at 650 nm. The survival rate was calculated as the percentage of optical density of culture in bile salt conditions compared to the optical density of culture without bile salts. All tests were carried out in triplicates.

Susceptibility to antibiotics of lactobacilli strains: Antibiotic susceptibility was tested by disk diffusion and by broth microdilution methods (CLSI, 2009a, 2009b).

The MIC (µg/ml) was defined as the lowest antibiotic concentration that resulted in no visible growth. For disc diffusion antibiotic susceptibility, Inhibition Zone Diameters (IZD) were measured and strains were classified as sensitive (IZD≥21mm), intermediate (16mm≤IZD≤20mm), and resistant (IZD≤15mm) according to interpretative standards defined by CLSI (2011) and Vlková *et al.* (2006). The MICs (µg/ml) were determined and the results of susceptibility status were interpreted according to the recent FEEDAP document of the European Food Safety Authority (EFSA) on the update of the criteria used in the assessment of antibiotics bacterial resistance of human or veterinary importance (EFSA, 2008) as well as epidemiological cut-off values defined by the ACE - ART Project results, ISO 10932 (2010). Strains showing MICs less than EFSA's breakpoints were considered sensitive; otherwise, they were resistant. The antibiotics including penicillin G, ampicilline, tetracycline, erythromycin, amoxicillin, chloramphenicol., obtained from Oxoid were tested:

Cholesterol assimilation of lactobacilli isolates: The ability of cultures to assimilate cholesterol was determined by a modified method described by Dora and Glenn (2002). The test medium for screening cultures for cholesterol uptake was sterile MRS broth containing 0.4% Bile salt N.3 and 0.01% cholesterol (polyoxyethanyl-cholesteryl Sebacate; Sigma). Strains were inoculated (1%) into 7ml volumes of the broth and incubated under anaerobic conditions for 24 h at 37°C. Bacterial cells were removed by centrifugation (4000 g, 10 min, 4°C). The spent broth and uninoculated control broth were then assayed for their cholesterol content by the enzymatic assay described by Salè *et al.* (1984). The dry weight of the cultures was determined after drying the centrifuged cells to a constant weight in an 80°C oven. All tests were carried out in triplicates.

RESULTS AND DISCUSSION

A total of 31 isolates of gram-positive and catalase-negative bacteria were isolated from cow's raw milk and purified on MRS medium supplemented with 0.5% (W/V) cystein-HCl. All the isolates were rod cells. Based on the criteria defined by Kandler and Weiss (1986) and Hammes *et al.* (1991), these isolates were assigned to the genus *Lactobacillus*. Lactic acid bacteria have been demonstrated to be responsible for the spontaneous lactic acid fermentation of dairy products in the Western Highlands of Cameroon, where three genera including *Lactobacillus*, *Enterococcus* and *Lactococcus* have previously been identified (Zambou *et al.*, 2004, 2008).

Because probiotic organisms should be acid and bile tolerant, acid and bile salt tolerance were examined to predict the survival of isolates in the gastrointestinal tract of live animals. 33 were selected for their tolerance to

Table 1: Surviving percentages of lactobacilli isolates incubated at 37°C after 6 and 24 h at pH 3 and 2

Isolates	Surviving percentage (%) at pH 3		Surviving percentage (%) at pH 2	
	After 6h	After 24h	After 6h	After 24h
1M	43.81	ND	ND	ND
2M	37.07	ND	ND	ND
3M	47.95	ND	ND	ND
4M	47.62	ND	ND	ND
5M	61.64	67.24	32.45	ND
6M	94.04	78.94	57.14	26.31
7M	29.62	ND	ND	ND
8M	73.80	58.20	44.28	ND
9M	74.48	56.15	40.44	ND
10M	84.81	85.96	50.64	50.01
11M	56.62	66.61	54.21	12.69
12M	63.10	76.27	58.33	23.72
13M	42.70	ND	ND	ND
14M	46.70	ND	ND	ND
18M	55.01	47.05	ND	ND
19M	35.71	ND	ND	ND
20M	42.50	ND	ND	ND
21M	56.81	71.64	50.00	43.28
35M	57.40	50.00	29.60	ND
36M	41.74	40.00	ND	ND
37M	67.74	54.84	31.30	ND
38M	62.62	62.50	34.90	ND
39M	59.52	50.00	26.19	ND
40M	26.02	19.44	ND	ND
41M	72.50	58.62	37.50	ND
42M	63.16	68.75	29.78	ND
43M	52.22	40.00	33.33	ND
44M	25.15	19.44	ND	ND
45M	42.05	23.53	ND	ND
46M	17.54	18.42	ND	ND
47M	44.00	37.14	ND	ND
48M	70.00	69.05	50.00	33.33
49M	74.07	63.41	40.74	ND
50M	40.00	14.28	ND	ND

ND: Not determined because, the isolates presented a surviving percentage <50% in the previous step. Such isolates were considered non acid tolerant and thus eliminated; Values in this table are means of three replicates.

acid. Their survival percentages at pH 3 and 2 are presented in Table 1. The data in this table show that the survival percentages varied within isolates and for the same isolate, it diminished with increasing in the acidity of the medium. The percentages also decreased with increasing incubation period. Indeed for all the isolates, the survival percentage after 6 h incubation was higher than after 24 h incubation. Bacteria used as probiotics are generally delivered to animals through their feed and thus, should have the ability to resist the stressful conditions in the intestinal tract, including acid and bile secretions (Gilliland, 1987). The pH in human stomach ranges from 1 (during fasting) to 4.5 (after meal), and food digestion can take up to 3h. Since *Lactobacillus* strains are known to survive at pH 4.6, which is also the final acidity of most fermented dairy products, lower pH values (2 and 3) were examined. Although all of the examined strains were resistant to pH 3 after 6 h of exposure, most of the strains displayed loss of survival when exposed to pH 2 for 6h. These results are in agreement with those obtained from

Table 2: Survival (log 10 cfu/mL) of selected lactobacilli isolates under acidic conditions after 5 h of incubation in citric acid

Isolates	Initial count (log ufc/mL)	Count after 5h (log ufc/mL)	Surviving percentage (%)
6M	9.20	6.00	65.21
8M	9.50	0.00	0.00
9M	9.00	0.00	0.00
10M	9.20	5.60	60.86
11M	8.80	5.90	67.04
12M	9.00	5.00	55.55
21M	9.10	5.75	63.18
48M	9.00	5.60	62.22
49M	9.30	0.00	0.00

Table 3: Surviving percentages of selected acid tolerant lactobacilli isolates incubated at 37°C for 6 and 24 h in 0.2 and 0.4% Bile salt N°3 (Oxoid)

Lactobacilli isolates	Surviving percentage (%) in 0.2 % bile salts		Surviving percentage (%) in 0.4 % bile salts	
	After 6 h	After 24 h	After 6 h	After 24 h
6M	93.11	83.87	77.01	67.74
10M	100.00	89.83	83.33	77.96
11M	90.69	74.13	77.90	72.41
12M	96.38	85.96	83.13	78.94
21M	95.34	93.44	90.69	80.32
48M	100.00	87.95	89.47	78.31

similar studies (Du Toit *et al.*, 1998; Jacobsen *et al.*, 1999; Dunne and Mahony, 2001; Maragkoudakis *et al.*, 2006).

The nine lactobacilli isolates demonstrating at least good tolerance under the acidic conditions using rapid selective method were screened for their ability to tolerate acidic condition in citric acid, pH 3 after 5 h (Table 2). Six of these isolates demonstrated high tolerance to acidic conditions of pH 3 after 5 h of exposure in citric acid at 37°C by showing surviving percentage greater than 50%. The highest resistance to acidic conditions was observed in isolate 11M with 67.04% of surviving percentage after exposure to pH 3. Three isolates (8M, 9M and 49M) lost their viability under exposure in citric acid pH 3 after 5h at 37°C. According to these criteria, six isolates (6M, 10M, 11M, 12M, 21M and 48M) were considered as acid resistant.

Table 3 shows the survival percentages of ten acid resistant isolates to 0.2 and 0.4% (W/V) of bile salts. All the isolates showed survival percentages >50%. The survival percentage decreased when the bile salts concentration increased. Survival percentages also decreased when the incubation period was increased. Usman (1999) stated that, for a strain to be considered as probiotic, it should be resistant in acidic conditions such as pH 3 and tolerant to 0.1% bile salts. In this study, the six isolates exhibited resistance to high acid conditions (6M, 10M, 11M, 12M, 21M and 48M) were also found to be bile-salts tolerant. The most tolerant strain was 21M. Bile-salt tolerance is important for strains to grow and survive in the upper small intestine where BSH (Bile-salt hydrolase) activity of such lactobacilli may play a role in the enterohepatic cycle (Maragkoudakis *et al.*, 2006).

Table. 4: Sensitivity of selected acid and bile salt tolerant lactobacilli isolates to some antibiotics by agar disc diffusion and microdilution methods

Antibiotics and concentration		Lactobacilli strains					
		6M	10M	11M	12M	21M	48M
Ampicillin (10 µg)	IZD (mm)	31±2	38±1	30±1	35±2	30±1	31±2
	MIC (µg/mL)*MIC BP = 2	0.5	0.5	0.5	0.5	0.5	0.5
Penicillin G (10 µg)	IZD (mm)	31±2	31±2	30±2	35±1	35±1	35±2
	MIC (µg/mL) *MIC BP = ND	2	2	2	2	2	2
Erythromycin(15 µg)	IZD (mm)	25±2	21±2	29±1	23±2	23±2	26±1
	MIC (µg/mL)*MIC BP = 1	0.25	0.25	0.25	0.25	0.25	0.25
Chloramphenicol (30 µg)	IZD (mm)	20±1	19±2	20±2	21±2	19±2	21±2
	MIC (µg/mL)*MIC BP = 8	2	2	2	2	2	2
Tetracycline (30 µg)	IZD (mm)	25±2	26±1	21±2	25±2	29±2	28±2
	MIC (µg/mL)*MIC BP = 32	8	8	8	8	8	8

*IZD: Inhibition Zone Diameters are means from triplicate determinations; Diameters of the discs are inclusive (6 mm); *: MIC BP: MIC Break points according to European Food Safety Authorities (µg/mL); ND: not defined breakpoint; MIC: Minimal Inhibitory concentration

Table. 5: Antimicrobial activity of selected acid and bile salt tolerant lactobacilli isolates against some pathogenic bacteria

Lactobacilli isolates	Antimicrobial activity of lactobacilli strains*			
	Staphylococcus aureus ATCC25922	Salmonella typhi ATCC6539	Pseudomonas aeruginosa ATCC27853	Escherichia coli ATCC 13706
6M	+	+	+	+
10M	+	+	+	+
11M	+	+	+	+
12M	+	+	+	+
21M	+	+	+	+
48M	+	+	+	+

*: The antimicrobial activity were determined by spot on the lawn test; +: Inhibition zone ≥2mm; ATCC: American Type Culture Collection

These isolates are promising with regards to their use as probiotics because of their ability to tolerate acidic conditions and bile salts.

Antibiotic susceptibility: The results of the antibiotic susceptibility tests are shown in Table 4. All the six isolates showed susceptibility to all the antibiotics tested. The isolates were more commonly sensitive to ampicillin and penicillin, which presented diameter of inhibition zones between 30 and 38 mm. The diameter of inhibition zones varied from 21 to 30 mm for erythromycin and tetracycline. With regard to MIC results, these isolates were sensitive to the protein synthesis inhibitors chloramphenicol, erythromycin and tetracycline as revealed by the comparison with the EFSA's breakpoints.

The smallest diameters of inhibition zones (21 to 29 mm) were observed with erythromycin and tetracycline. The resistance of *Lactobacillus spp.* from human gut or isolated from dairy origin to erythromycin were previously reported (Ahn *et al.*, 1992). According to their diameter of inhibition zones and their MIC values lower than the breakpoint, all the strains tested were susceptible to ampicillin, a cell wall synthesis inhibitor.

Antibiotic susceptibility of lactobacilli is one of the crucial criteria for the safety point of view of potential probiotics since bacteria used as probiotics may serve as host of antibiotic resistance genes, which can be transferred to pathogenic bacteria (Scott, 2002; Temmerman *et al.*, 2002; Mathur and Singh, 2005). The antibiotic resistance of pathogenic bacteria is an

increasing medical problem. Therefore, the antibiotic susceptibility test therefore should be incorporated for the safety assessment of the desired property of the promising probiotic lactobacilli. Our isolates were least sensitive to chloramphenicol. Previous studies also confirm the generally lower resistance of lactobacilli species towards this antibiotic (Temmerman *et al.*, 2002; Magkoudakis *et al.*, 2006). Since the isolates tested in this work were not resistant to any of antibiotics, they could be safe for use by animals and eventually by humans, and their lack of resistance also indicates that they may not contribute to the transfer of resistance to other microorganisms. The smallest diameters of inhibition zones (21 to 29 mm) were observed with erythromycin and tetracycline. According to their diameter of inhibition zones and their MIC values lower than the breakpoint, all the strains tested were susceptible to ampicillin, a cell wall synthesis inhibitor.

Detection of antibacterial activity: In this study, *Staphylococcus aureus*, *Salmonella typhi*, and *Escherichia coli* were used as the test bacteria because they are occasionally found as food borne microorganisms that might cause gastroenteritis. The results revealed that the antibacterial activity of the six selected lactobacilli could inhibit all pathogenic bacteria tested, however at different inhibition levels as shown in Table 5. Of the nine isolates tested, six (6M, 10M, 11M, 12M, 21M, 48M) inhibited the growth of all the pathogens used as indicators. The good probiotics should present their antimicrobial actions particularly to the pathogens in the

Table 6: Cholesterol uptake of selected acid and bile salt tolerant lactobacilli isolates in MRS broth supplemented with 0.4 % bile salts and 0.01 % cholesterol

	Lactobacilli isolates					
	6M	10M	11M	12M	21M	48M
Cholesterol uptake (mg)	0.48±0.04 ^a	0.79±0.05 ^a	1.05±0.04 ^b	0.72±0.01 ^a	0.92±0.02 ^a	0.65±0.00 ^a
Cellular weight (mg)	30±2	40±5	40±6	20±4	20±3	30±4
CA (mg of cholesterol per g of cells)	15.66±1.33 ^a	20.35±1.35 ^a	26.01±0.99 ^c	38.05±0.65 ^b	45.21±0.8 ^b	21.96±0.30 ^a

CA: cholesterol assimilation; each value represents the average of the three trials; ^{abc}: In the same lines; values followed by different superscript letters differ significantly (p<0.05)

Table 7: Physiological and biochemical characteristics of selected lactobacilli strains showing some promising probiotic properties

Characteristics	Lactobacilli strains (n = 6)					
	6M	10M	11M	12M	21M	48M
Growth at						
10°C0	+	+	+	+	+	+
45°C0	+	-	+	+	+	-
Production of CO ₂ from glucose	-	-	-	-	-	-
Esculin hydrolysis0	+	+	+	+	+	+
Production of acid from:						
Glycerol	-	-	-	-	-	-
L-arabinose	-	+	-	-	-	+
Ribose0	+	+	+	+	+	+
D-xylose	-	+	+	+	+	+
Galactose0	+	-	-	-	-	+
D-Glucose0	+	+	+	+	+	+
D-Fructose0	+	+	+	+	+	+
D-Mannose0	+	+	+	+	+	+
Rhamnose	±	-	±	±	±	-
Inositol-	-	-	-	-	-	-
Mannitol0	+	+	+	+	+	+
Sorbitol0	+	+	+	+	+	+
Methyl D-glucoside0	+	+	+	+	+	+
Methyl D-mannoside0	+	+	+	+	+	+
NAcetylglucosamin0	+	+	+	+	+	+
Amygdalin0	+	+	+	+	+	+
Arbutin0	+	+	+	+	+	+
Salicin0	+	+	+	+	+	+
Cellulose0	+	+	+	+	+	+
Maltose0	+	+	+	+	+	+
Lactose0	+	+	+	+	+	+
Mellibiose0	+	+	+	+	+	+
Sucrose0	+	+	+	+	+	+
Trehalose0	+	+	+	+	+	+
Inuline	-	+	-	-	-	+
Melezitose0	+	+	+	+	+	+
Raffinose0	+	+	+	+	+	+
Starch	-	-	-	-	-	-
Gentibiose0	+	+	+	+	+	+
Turanose0	+	+	+	+	+	+
D-arabitol	-	-	-	-	-	-
Gluconate0	+	+	+	+	+	+
5cetogluconate	-	-	-	-	-	-
Tagatose	-	-	-	-	-	-
	Lb plantarum (79,9%)	Lb plantarum (99,9%)	Lb plantarum (69%)	Lb plantarum (75,9%)	Lb plantarum (49,9%)	Lb plantarum (99,9%)
Identification	Lb rhamnosus (17%)		Lb rhamnosus (27%)	Lb rhamnosus (15%)	Lb rhamnosus (65%)	Lb plantarum (99,9%)

Only the sugars producing acid by at least one strain are reported in this table; +: positive reaction; -: negative reaction; The percentage following the scientific names of strains represent the percentage of similarity from the computer-aided database API LAB Plus V3.2.2

GI system. Probiotics have been used as growth promoters to replace the widely used antibiotics and synthetic chemical feed supplements (Fuller, 1991). This has been made possible by their ability to produce

antimicrobial substances towards pathogens. The inhibitory activity of lactobacilli strains against either gram-negative or gram-positive pathogens has been found to be very variable. This inhibitory activity varies also

within strains (Maragkoudakis *et al.*, 2006). Lactobacilli are highly competitive largely due to their production of several antimicrobial compounds such as organic acids, hydrogen peroxide, reuterin and bacteriocins (Kaktcham *et al.*, 2011). The six isolates (6M, 10M, 11M, 12M, 21M, 48M) that exerted antimicrobial effects on the growth of all pathogenic strains were retained as potential probiotic candidates and screened for the assimilation of cholesterol.

Assimilation of cholesterol: The amount of cholesterol assimilated during 24 h of anaerobic growth at 37°C (Table 6) revealed a wide variation among isolates. All isolates examined were able to assimilate cholesterol to some extent. The amounts of cholesterol assimilated by cultures ranged from 15.66 mg/g of cells (6M) to 45.22 mg/g of cells (21M). These values are not significantly different among the isolates 11M and 48M ($p>0.05$) or among isolates and 12M. Isolates 6M, 10M, 11M and 21M are significantly different from each other based on their ability to assimilate cholesterol *in vitro* ($p<0.05$). Hypercholesterolemia is a major risk factor associated with coronary heart diseases, and it is considered that keeping blood cholesterol at a desirable level is one of the major preventive strategies for these diseases. Previous data from *in vitro* studies showed that some strains of *Lactobacillus* are able to take up cholesterol into their membrane (Dambekodi and Gilliland, 1998; Lin and Chen, 2000). Some natural microorganisms in human intestine are beneficial in terms of lowering serum cholesterol level by breaking down the bile salts (Fernandez *et al.*, 2003; Lim *et al.*, 2004). The results presented in this work are partly similar to the data reported by some authors (Gilliland, 1987; Dora and Glenn, 2002) in that some strains of *Lactobacillus* are able to remove cholesterol from culture medium during anaerobic growth in the presence of bile salts. Previous studies have shown that, in order to assimilate cholesterol, the organisms must be able to grow in the presence of bile (Dora and Glenn, 2002). In this work (Table 6), all the isolates tested were able to assimilate cholesterol *in vitro*; but isolate 21M was the best candidate since it consumed 45.21 mg of cholesterol per g of cells (dry weight).

The six potential probiotic strains were identified as shown in Table 7 using their phenotypic characteristics as describe by Kandler and Weiss (1986) and Hammes *et al.* (1991). Four gram-positive and catalase-negative isolates (6M, 11M, 12M, 21M) grew at 45°C. All isolates grew at 10°C. None of the six isolates produced CO₂ from glucose. These characteristics suggested their classification as facultative heterofermentative lactobacilli. In addition, due to their ability to grow at 10°C and their inability to grow at 45°C, isolates 10M and

48M were considered as mesophilic lactobacilli which according to Bergey's classification can be grouped in the genus *Lactobacillus* Group II (Sneath *et al.*, 1986).

The fermentative profiles were strains dependent (Table 7). All isolates hydrolyzed esculin and produced acid from D-ribose, hexoses (D-glucose, D-fructose, D-galactose and D-mannose) and the disaccharide (sucrose, lactose and maltose). The acid production from L-Arabinose D-rhamnose and inulin depends on the individual isolates. Fermentation profiles of isolates 10M and 48M were similar and have a 99% similarity with the type strain of the species *Lactobacillus plantarum*. This identification is consistent with the phenotypic characteristics described by Kandler and Weiss (1986) showing that these two isolates have the ability to grow at 10°C and not at 45°C. Moreover, isolates 6M, 11M, 12M, 21M from different samples of cow's milk have similar fermentation profiles. These profiles resemble the type strains of either species samples of cow's milk have similar fermentation profiles. These profiles resemble the type strains of either species *Lactobacillus plantarum* or *Lactobacillus rhamnosus*. Zambou *et al.* (2008) demonstrated that strains of *Lactobacillus plantarum* constituted the dominant species of lactobacilli present in dairy products from western highland region of Cameroon.

It was shown that the species of the genus lactobacilli group II are facultative heterofermentative lactobacilli as they ferment hexoses exclusively by producing lactic acid, but also ferment pentoses after induction of phosphoketolase with lactic acid and acetic acid production. Lactobacilli Group II consist of three complex species based on their DNA-DNA homology and several species showing no phylogenetic relationship. The first complex includes *Lactobacillus plantarum* sp. and genetically close species such as *Lactobacillus pentosus*, *Lactobacillus graminis* and *Lactobacillus agilis*. Using DNA-DNA hybridization, Dellaglio *et al.* (1975) have shown that the species *Lactobacillus plantarum* are in the same group with *Lactobacillus pentosus*. Species *Lactobacillus paracasei* ssp. *paracasei* and *Lactobacillus rhamnosus* derive from the complex designated "casei Group" which has long consisted of mixed species whose status and relationships should be clarified. The use of physiological tests and fermentation of sugars has long been successfully applied in the identification of lactic acid bacteria (Bille *et al.*, 1992). This identification method does not always differentiate between species with similar phenotypic but genetically distant properties. Thus, before proceeding with the selection of bacteria on the basis of their many probiotic properties, we proceeded to confirm phenotypic identification by the Rep-PCR technique.

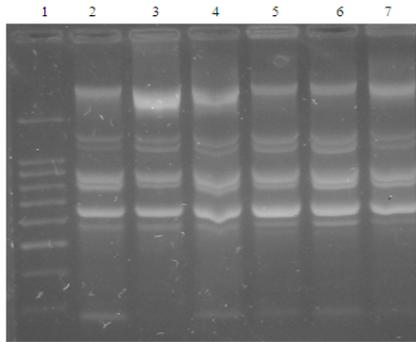


Fig. 1: Illustration of PCR identification of lactobacilli by Rep-PCR: Starting from the left side: lane 1: the 1Kb DNA Ladder; lane 2-7: Lactobacilli isolate 6M, 10M, 11M, 12M, 21M and 48M

After the basic phenotypic characterisation of the six strains, they were identified to species level by Rep-PCR. BOXA1R-PCR banding patterns as shown in Fig. 1. All the selected isolates had similar but not identical Rep-PCR profiles. These presence of many DNA bands in the profile of each isolate indicate that highly conserved repetitive DNA elements, such as BOX elements, seem to be widely distributed in the genomes of our isolates. The amplification of the sequences between each of these repetitive elements has been used to generate DNA fingerprints of several Gram-negative and Gram-positive bacterial species (De Urraza *et al.*, 2000; Mohammed *et al.*, 2009). By comparing these profiles with those of the type reference strains of *Lactobacillus paracasei ssp paracasei*, *Lactobacillus plantarum* and *Lactobacillus rhamnosus*, the dendrogram in Fig. 2 shows that our

isolates were very similar to *Lactobacillus plantarum*. The repetitive BOX elements have generated typical DNA fingerprints of each isolate allowing their rapid and reliable speciation. The presence of *Lactobacillus plantarum* in raw milk was in accordance with previous results. Identification at species level was necessary for further testing and possible application of strains as probiotics.

CONCLUSION

In this study, six lactobacilli strains identified as *Lactobacillus plantarum* were found to possess some preliminary probiotic activities such as tolerance to acidic conditions and to bile salts, sensitivity to antibiotics, production of antimicrobial effects and ability to assimilate cholesterol. These strains are good candidates for further investigation under *in vitro* as well as *in vivo* conditions to elucidate their potential beneficial health effects and their possible application as novel probiotic strains in the food industry.

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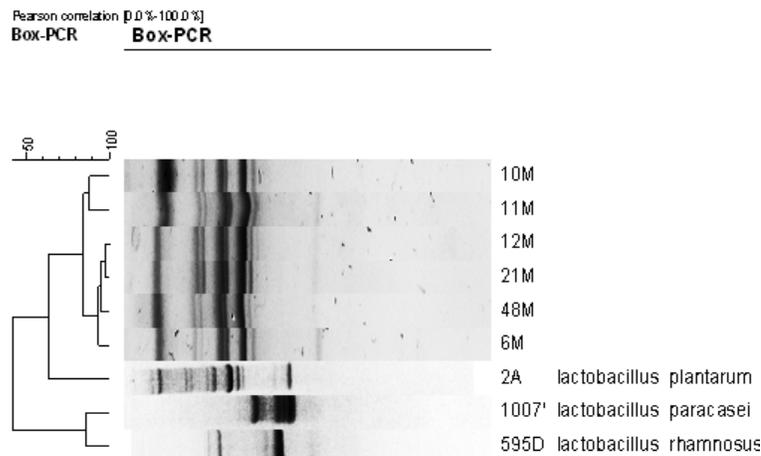


Fig. 2: Rep-PCR fingerprints of reference strains *Lb. plantarum* (2A), *Lb. paracasei* (1007'), *Lb. rhamnosus* (595D) and selected lactobacilli strains (6M, 10M, 11M, 12M, 21M, 48M) from raw milk and generated dendrogram from combined fingerprints. The dendrogram was constructed using the unweighted pair group method using arithmetic averages with correlation levels expressed as percentage

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