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Rep PCR identification and Functional properties of Lactic Acid Bacteria Isolated from fermented Cows' Milk in Algeria

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***Abstract*— Milk and indigenous fermented, milk based products are a rich source of lactic acid bacteria (LAB) and may serve as a source of potential probiotics. In the present study LAB were isolated from raw cows' milk sampled at five different Algerian farms. A total of 24 Gram positive, catalase negative isolates where identified to species level using a combination of (GTG) 5-based rep-PCR fingerprinting and 16S rRNA gene sequencing. All isolates were identified as *Enterococcus faecium* and all isolates had highly similar rep-PCR profiles. Four representative isolates were screened for acid tolerance, bile salts tolerance, antimicrobial susceptibility, antibacterial activity and haemolysis. The four selected strains all exhibited good tolerance to low pH (2, 3, and 4), and to bile salts (concentrations of 0.5%, 1%, and 2%) and were susceptible to majority of antibiotics tested and exhibited resistance only to ofloxacin, erythromycin, pefloxacin. Cell-free supernatants of the four tested strains all inhibited *Staphylococcus aureus, Escherichia coli* and *Listeria monocytogenes* but not Salmonella Typhi. No haemolytic activity was observed.**

***Keywords*— LAB, rep PCR, row cow's, Assessment food, Technological characterisation.**

I. INTRODUCTION

Lactic acid bacteria (LAB) are contributing to texture and flavour development of a wide range of fermented foods in addition to enhancing microbial safety and shelf-life [1], due to their production of lactic acid, acetic acid, H2O2, bacteriocins and diacetyl [2].

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. Desired characteristics include e.g. tolerance to acid and bile, cholesterol-lowering potential, ability to hydrolyze bile salt, being non-hemolytic, ability to possess antimicrobial properties and lack of antibiotic resistance [3].

Cow's milk is widely consumed by Algerians (also more than goat and sheep milk) either as raw milk products or as traditional fermented types. Both raw cow's milk and indigenous fermented milk products are good sources of LAB[4].

Therefore, the objectives of this study were to isolate LAB from raw cow milk and investigate potentially important characteristics such as acid and bile tolerance, antibiotic resistance profile[5], and inhibition of known pathogens as a first step towards developing probiotics based on Algerian, indigenous milk-related isolates.

II. MATERIALS AND METHOD

A. Sample Collection

Five raw cow milk samples were collected in sterilized bottles from different cow farms in Algeria. The samples were kept in ice boxes transported to the laboratory of Food and Industrial Microbiology, University of Oran Ahmed Ben Bella, Algeria.

B. Isolation of lactic acid bacteria

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Milk samples were incubated at 37°C until coagulation. Coagulated samples were 10-fold diluted, plated on MRS agar [6], and incubated anaerobically at 37 °C for 48 h. Sixty colonies with different morphologies were subjected to Gram stain and catalase test. 24 Gram-positive and catalase-negative isolates were subjected to further studies. Glycerol stock of LAB isolates were prepared and stored at -80 °C. Prior to use ,the purified cultures were activated by sub-culturing twice (37 °C, 24 hours) in MRS broth before use[7].

C. Identification of strains

C.A.1 Phenotypic Characterization

Phenotypic characterisation of the LAB strains were performed as described previously[8].Provisional, tentative identification was made by Gram staining, cell morphology and catalase reaction. Further characterization such as production of gas from glucose, growth at different temperature (10, 15, and 45°C) and pH 9,6 as well as the ability to grow in different concentrations of NaCl (2%, 4% and 6.5%.w/v), Shermans test and survival after heating to 60°C for 30 min were carried out[9]. Further, hydrolysis of arginine and esculine [10], and production of acetoin from glucose, as determined by using the Voges-Prokauer test and the ability to ferment cellebiose, galactose, mannitol, melizitose, melibiose, ribose, trehalose, xylose, glucose, lactose, saccharose, fructose and arabinose were carried out[11].

C.A.2 Genotypic characterization.

C.A.2.1 DNA Extraction for PCR and sequencing Reactions

Overnight-cultures of each strain were grown at 37°C in MRS broth. Cells were harvested by centrifugation (12,000 rpm, 1 min). Genomic DNA was extracted using the Instagene Matrix Kit (Bio-Rad Laboratories), following the instructions of the manufacturer. Extracted DNA was stored at -20°C until use.

C.A.2.1.1 Rep-PCR

Rep-PCR fingerprinting using the primer (GTG)5 (5'-GTG GTG GTG GTG GTG-3') (DNA Technology A/S, Denmark) was carried out following the protocol of [12]. The rep-PCR profiles were analysed using the Bionumerics software package (Applied Maths, Sint-Martens-Latem, Belgium) as previously described [13].

C.A.2.1.2 16S rRNA gene sequencing

The 16S rRNA gene was amplified using the universal primers 27F and 1540R[14],under the following conditions: initial denaturation at 95°C for 5 min, 35 cycles at 95°C for 30s, 60°C for 30s and 72°C for 120s, followed by a final extension at 72°C for 10 min. PCR products were sent to a commercial sequencing facility (Macrogen, South Korea). Sequences were manually corrected and assembled using CLC Genomics Workbench 8.0 (Aarhus, Denmark). Subsequently, the corrected sequences were aligned to 16S rRNA gene sequences in the GenBank database using the BLAST algorithm [15] . The nucleotide sequences obtained in this study have been assigned GenBank Accession Nos.

D. Technological charcterisation

A. *Acid tolerance*

Resistance to acid conditions was determined according to [8]. Isolates were cultivated anaerobically in MRS at 37°C, the cells were centrifuged (3000 x g, 10 min) and washed twice with sterile saline (0.85% NaCl), and re-suspended in 10 ml of MRS broth. Cell suspension (1% of total volume) was inoculated into 10 ml of MRS with pH adjusted to 2.0, 3.0 or 4.0 using 6 N hydrochloric acid (HCl). The cultures were then anaerobically incubated at 37°C for 3 hours. At the end of incubation, the viable cells were enumerated by pour plate counts on MRS agar.

B. *Bile tolerance*

The ability of the isolates to grow in the presence of bile was determined according to the method of [16], with some modifications. A suspension of 100 ml of cells was collected by centrifugation (3400×g, 10 min), washed twice in saline (8.5 g NaCl/L) and resuspended in 10 ml MRS broth with 0.5, 1% or 2% of oxgall (Sigma-Aldrich, MD, USA). At the end of incubation at 4 hours, viable cell counts were examined on a MRS agar plate using the spread plate technique. Experiments of acid and bile tolerance were repeated in triplicate.

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C. *Antimicrobial activity*

The ability of the isolates to inhibit Listeria monocytogenes (ATCC 7659), Escherichia coli (ATCC 25955), Staphylococcus aureus (ATCC 7153) and Salmonella Typhi (ATCC 25925) was examined by the agar disc diffusion test [11]. 100µl of each pathogen was suspended in 4ml sterile water, approximately (108 CFU/mL)and applied onto PCA medium until absorption, after that, a sterile paper disc (5 mm) moistened with 20µl of cell free supernatant (obtained by centrifugation (2500×g/10 min and neutralized to pH 6.5 ± 0.1) of each strain tested was added. Susceptibility of the indicator pathogens to the cell free supernatants was assessed by measuring the zone of inhibition of bacterial growth around the discs (radius - mm) after incubation for 24 h at 37 ºC. A clear zone of inhibition of at least 1 mm radius was recorded as positive [17]. The experiment was performed in triplicate.

D. *Antibiotic susceptibility*

The determination of susceptibility profile to antibiotics was determined as described by[16],with the uses of discs of antibiotics containing either chloramphenicol (30 µg), erythromycin (5 µg), ofloxacin (5 µg), tetracycline (30 µg), vancomycin (30 µg), Gentamicin (10 µg), cefriazone (5 µg), pefloxacin (5 µg),Amoxicillin (25 µg), Cotrimaxazole (25 µg) and Streptomycin(10 µg),ciprofloxacin (10 µg) (Oxoid, UK). The tested isolates were propagated as lined out above and suspended in 4 mlsterile distillate water in order to achieve 0.5 McFarland turbidity standard, then the inoculums was spread onto MRS, after which the antibiotic disks was applied to the plates. After incubation for 24 h at 37°C, the zone of inhibition was measured and expressed as millimeter (mm). The experiments were performed in triplicate.

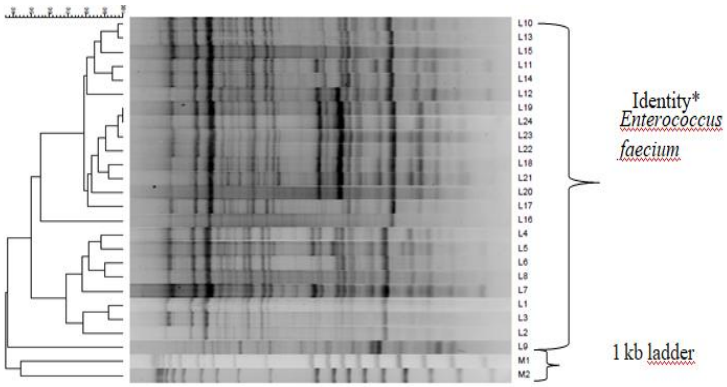
E. *Hemolytic activity*

The tested isolates were examined on Colombia blood agar after incubating in MRS broth at 37°C for 15 h. The hemolytic reaction was evaluated by observing both partial hydrolysis of red blood cells and the production of a green zone (α-hemolysis), as well as the total hydrolysis of red blood cells producing a clear zone around the bacterial colony (γ-hemolysis) or no reaction (β-hemolysis)

III. RESULTS

A total of 24 gram-positive, catalase negative coccoid isolates obtained from MRS were presumptively considered as LAB (Gram positive, catalase negative coccoid cells). The presumptive LAB isolates were clustered by (GTG) 5-based rep-PCR fingerprinting, resulting in only one cluster, as shown in Fig 1.

The isolates were identified by 16SrRNA sequencing Representative isolates were selected for sequencing of the 16S rRNA gene followed by BLAST search at GenBank database. High similarities (99 –100 %) between the isolates obtained during the present study and sequences deposited in GenBank were found . All isolates were identified as Enterococcus faecium. All isolates belonging to this group grew at 45°C as well as 10°C, and in media containing 6.5 % of NaCl, or with pH 9.6 and did not hydrolyze arginine and esculine. All had a broad carbohydrate fermentation pattern fermenting fructose, glucose, lactose, and mannitol, trehalose, amidon, sucrose and melibiose while not fermenting sorbitol, glycogen, rhamnose and melezitose.



IV. Figure 1: The dendrogram is based on Dices's Coefficient ofsimilarity with the unweighted pair group method with arithmetic averages clustering algorithm (UPGMA).

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Four isolates of E. faecium were subjected to a range of tests with the aim of assessing traits of potential importance for further investigations into probiotic properties.

The isolates exhibited tolerance to acidic conditions (Table 1), though pH 2 were detrimental to all 4 strains. Isolates L11 and L13 retained the same level of viability after 3 hours at pH 3. When exposed to bile salts all isolates exhibited reduction in viability, with the exception of Isolate L9, which demonstrated high tolerance (Table 2). L11, L9, L13 demonstrated a high susceptibility to vancomycin and chloramphenicol and moderate susceptibility to tetracycline, ampicillin, gentamicin and streptomycin, L20 was moderately suseptible to Chloramphenicol, tetracycline, vancomycin, gentamycin, erythromycin and streptomycin and normal susceptible to ofloxacin, ampicillin, Cotrimaxazole, for the resistance to antibiotics tested L11, L13, L20,L9 were resistance to ofloxacinerythromycin, pefloxacin [18] (Table 3).

Furthermore, the selected isolates were screened for their antimicrobial activity using agar disc diffusion. All 4 tested isolates showed strong inhibitory activity against E.coli and S. aureus while only L11 and L13 were active to Listeria monocytogens and against both L. S. Typhi. Isolate L 9 also showed modest activity against L. monocytogenes (Table 4). The four tested isolates showed no hemolysis of sheep blood.

TABLE 1

ACID TOLERANCE OF TESTED ENTEROCOCCUS FAECIUM AFTER EXPOSURE TO ACIDIC CONDITIONS (PH 2, 3 AND 4) DURING 3 H OF INCUBATION AT 37°C.

Isolate codes	pH2		pH3		pH4	
	0h	3h	0h	3h	0h	3h
L11	9.13 ± 0,01	00 ± 0.00	8.96a ± 0, 01	8 .18a + 0.01	9.25a ± 0.04	9.18a+ 0.05
L13	9.20 ± 0,05	00 ± 0.00	9.15b ± 0, 01	8.44b + 0.05	9.15a ± 0.30	9.06a± 0.06
L 20	8.53 ± 0,04	00 ± 0.00	8.56c ± 0, 01	8.04c ± 0.06	8.58b ± 0.10	7.98b± 0.01
L 9	9.10 ± 0,05	00 ± 0.00	9.17b ± 0, 01	8.67d ± 0.02	9.18a ± 0 .17	8.65c± 0.01

[abcd] Means in the same column followed by different superscript letters are significantly different (P< 0.05). L11: *Enterococcus faecium* (N11); L13*: Enterococcus faecium*; L 9: *Enterococcus Faecium* (N9); L20: *Enterococcus faecium* (N20).

TABLE 2

BILE TOLERANCE OF TESTED *ENTEROCOCCUS FAECIUM* AFTER EXPOSURE TO BILE SALTS CONDITIONS (0.5%, 1% AND 2%) DURING 3 H OF INCUBATION AT 37° C.

Isolate codes	0.5%		1%		2%	
	0h	3h	0h	3h	0h	3h
L11	9.16a ± 0.01	8.96a ± 0.01	9.22a ± 0, 01	7 .50a ± 0.03	9.21a± 0.01	9.18a ± 0.01
L13	9.22a ± 0.05	9.41b ± 0.01	9.15a ± 0, 01	8.42b ± 0.02	8.10a± 0.05	8.18a ± 0.01
L 20	8.58b ± 0.04	8.52c ± 0.02	8.58b ± 0, 01	8.04c ± 0.03	8.41b± 0.02	7.95b ± 0.03
L 9	9.07c ± 0.05	9.06a ± 0.04	9.18a ± 0, 01	8.67d ± 0.04	9.14a± 0 .02	8.71c ± 0.06

[abcd] Means in the same column followed by different superscript letters are significantly different (P< 0.05).L11: *Enterococcus faecium* (N11); L13:*Enterococcus faecium*; L 9: *Enterococcus Faecium* (N9); L20: *Enterococcus faecium* (N20).

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TABLE 3.1

ANTIMICROBIAL SUSCEPTIBILITY TESTING OF ENTEROCOCCUS FAECIUM STRAINS

Isolate codes	Tests of susceptibility				
	CO	GE	PE	ER	S
L11	S (++)	S (++)	S (++)	R (-)	S (++)
L13	S (+)	S (++)	R (-)	S (++)	S (++)
L 20	S (++)	S (++)	S (+)	S (++)	S (++)
L 9	S (+)	S (++)	R (-)	R (-)	S (++)

(-) no inhibition, (+) inhibition zone 0.1 to 1.0 mm; (++) inhibition zone 1.1 to 2.0 mm; (+++) inhibition zone > 2.1 mm. L11: Enterococcus faecium (N11); L13: Enterococcus faecium; L 9: Enterococcus Faecium (N9); L20: Enterococcus faecium (N20).

TABLE 4: ANTIMICROBIAL ACTIVITY OF CULTURE SUPERNATANTS FROM STRAINS OF

ENTEROCOCCUS FAECIUM AND 4 PATHOGENIC AND SPECIFIC SPOILAGE MICRO-ORGANISMS

Isolate codes	Zone of inhibition (mm)			
	S. areus	E. Coli	L. Monocytogenes	S. Typhi
L11	+++	+++	++	+
L13	++	++	+	+
L20	++	++	-	-
L9	++	++	+	-

(-) no inhibition, (+) inhibition zone 0.1 to 1.0 mm; (++) inhibition zone 1.1 to 2.0 mm; (+++) inhibition zone > 2.1 mm. L11: Enterococcus faecium (N11); L13: Enterococcus faecium; L 9: Enterococcus Faecium (N9); L20: Enterococcus faecium (N20).

V. DISCUSSION

This study focused on isolation and identification of lactic acid bacteria from spontaneously fermented raw cow's milk. All isolates were identified as E. faecium with high certainty. Interestingly, all isolates had highly similar rep-PCR-profiles indicating high interrelatedness between the isolates even though they were obtained from milk from 5 different farms. E. faecium has many traits that makes it suitable as probiotic. Especially the rather broad-spectrum antimicrobial activity against pathogens makes them interesting.

However, many E. faecium strains are resistant to a wide range of antibiotics and some strains have been implicated as opportunistic pathogens, making it essential to properly characterize the isolates, if they are to have potential use a probiotics in the future [19]. Consequently, a range of tests to establish fundamental traits important for putative probiotics were investigated. Further, acid and bile salts tolerance are important traits if a given putative probiotic strain is to survive stomach, which has pH varying between 2 and 4 and small intestine passage with a concentration between 0.2 and 2% of bile salts [20].

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The four tested isolates showed equal survival at pH 3 and pH 4, but none survived pH 2. These results are in agreement with the findings of [21]. They reported that the viable counts of Enterococcus were significantly affected by pH 2.

For bile salts tolerance, all four isolates retained viability, though with a decreasing tendency during 3 hour of incubation, Similar observations have been made in other studies by [22],who observed that the Enterococcus faecium can survive up till 3.5% of bile salt. It has been argued, that the high tolerance to acid and bile salts by Enterococcus faecium is due to the commensalism of this type of bacteria in the gastrointestinal tract of human and animals [23] .

Excessive use of a combination of nonprescription antibiotics usage has resulted in the emergence of multiple high resistant strains of Enterococci to these antibiotics especially chloramphenicol and vancomycin (Yousif et al. 2005). According to the findings of Mathur and Singh(2005), and (Banwo2012) , the E .faecium can consider a probiotic for using animal and silage inoculants additionally to humans, there was existed since the pre-antibiotic era and is susceptible to erythromycin (15µg), streptomycin/penicillin (streptopen35µg), gentamicin (10µg), chloramphenicol (30 µg) and tetracycline (30 µg). It also possesses intrinsic resistance to kanamycin (30 µg), streptomycin (10 µg) and oxacillin (5 µg) (Mathur and Singh 2005).Enterococcus faecium has proved moderately sensitive some antibiotics including streptomycin, gentamicin, however, it was showed that E.faecium L11, L9, L13 proved high susceptibility to vancomycin and chloramphenicol, while L20 was moderately suseptible to this antibiotics, these results are in similar compared to the investigation of the Mathur and Singh 2005 for the resistance characteristics of probiotics E. faecium and accordance to the finding of (Banwo 2012) [24]were screened the high sensitively of E. faecium CM4 to vancomycin and moderately to Gentamicin. In other study to Enterococcus isolated from chees and dairy milk was confirmed the resistance of this strains to vancomycin[25], these in intrinsically resistant ranging between, the low levels and a high-level to gentamicin [26].

The antimicrobial activity of the four E. faecium strains is likely attributed to compounds produced during including metabolites, organic acids, and bacteriocins [27]. All four strains showed good activity against E. coli and S. aureus, while L. monocytogens and S. Typhi were only inhibited to a smaller extent. All in all these findings support that the isolated E. faecium strains might have potential a probiotics in the future.

Lack of hemolytic activity is considered an important safety aspect for the selection of strains intended for human use(Staff et al. 2002)[28]. None of the four tested Enterococcus faecium strains hydrolyzed sheep blood (γ-haemolysis) in vitro.

VI. CONCLUSION

A total of 24 LAB isolated from row cow milk were characterized by phenotypic techniques and genotypic techniques using rep-PCR and 16S rRNA gene sequencing. All isolates were identified as Enterococcus faecium. Further, LAB isolated from cow milk exhibited some desirable probiotic properties in vitro, such as antimicrobial activity, bile and acid tolerance. More investigations may be warranted to elucidate its potential health benefit and its application as promising probiotic strain in fermented food and in the feed industry.

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