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Probiotic behavior study of four *Enterococcus faecium* isolated from raw milk in Algeria

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Abstract

Lactic acid bacteria (LAB) were isolated from spontaneously fermented raw goat milk collected from Relizane, Algeria, and assessed for their probiotic properties. Four acid-tolerant strains were selected. By using 16S rDNA sequencing, these isolates were confirmed to be *Enterococcus faecium*. The strains exhibited good survivability in a highly acidic environment at pH 2 and in bile salts at concentrations of 0.3% and 2%. The strains were susceptible to antibiotics except colistin and were found to inhibit the growth of *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes* and *Salmonella* Typhi. The strains were nonhemolytic and showed weak production of exopolysaccharides (EPSs). The study confirmed that all the identified strains have in vitro probiotic properties.

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Introduction

Enterococcus species, which belong to the family of lactic acid bacteria (LAB), are ubiquitous microorganisms that constitute a large proportion of the autochthonous microflora, ranging from the mammalian gastrointestinal tract to foods and food products (Belgacem *et al.,* 2010). A variety of microorganisms, typically food-grade LAB, have been evaluated for their probiotic potential and are applied as adjunct cultures in various types of food products or in therapeutic preparations (de Almeida Júnior *et al.,* 2015). Furthermore, *Enterococcus* species harbor some useful biotechnological and functional properties, including contributing to the ripening and flavor enhancement of several types of food, such as cheeses and sausages, and to the production of antimicrobials (Banwo *et al.,* 2013), and have been used as probiotics, which may improve the microbial balance in the intestine or may be used in the treatment of gastroenteritis in humans and animals (Pieniz *et al.,* 2014).

According to (on Food Additives. Meeting and Organization 2002), probiotics are live microorganisms with proven beneficial functions in the host when ingested in sufficient quantities. Promising probiotic strains, which include members of the genera *Lactobacillus*, *Bidobacterium*, and *Enterococcus*, are confirmed to have these probiotic functions (Pieniz *et al.,* 2014). There should be several criteria in order to use microorganisms as probiotics, including tolerance to acidic and bile conditions, cholesterol-lowering potential, the ability to hydrolyze bile salts, non-hemolytic properties, antimicrobial properties, and the ability to survive during the fermentation process (Ahmadova *et al.,* 2013). Various studies have revealed that probiotics can prevent or alleviate symptoms of inflammatory bowel disease, irritable bowel syndrome, constipation, antibiotic-associated and acute diarrhea, hypertension, and diabetes (Nueno-Palop and Narbad 2011). Functional foods have several therapeutic benefits, including antihypertension, anticancer, and hypoglycemic properties and antioxidant and immunomodulatory effects (Rehaiem *et al.,* 2014). The objectives of this work were to evaluate four *Enterococcus faecium* strains isolated from Algerian spontaneously fermented raw goat's milk and for potential use as probiotic strains with regard to their antimicrobial activity, their antibiotic susceptibility, their ability to survive under acidic and bile salt conditions, their hemolytic activity and their exopolysaccharide (EPSs) production ability and to use 16S rRNA sequencing to identify the strains.

Materials and methods

Sampling, isolation, and cultivation of bacterial strains

The five samples of milk analyzed in this study were obtained from five different farms in the province of Relizane, Algeria. For all samples, 1ml of milk was added to 9ml of sterile physiological saline and homogenized for 5 min. Appropriate dilutions were plated onto M17 agar (Difco, USA) and incubated anaerobically at 42°C for 48-72 h. Colonies were randomly selected from M17 agar containing less than 300 colonies and purified on M17 agar. The isolates were presumptively identified by the following tests: observation of colony characteristics and cell morphology, Gram staining, catalase, growth at 15°C and fermentation of sugars. After purity control, the cultures were maintained in MRS broth.

Identification of selected isolates by 16S rDNA

16S rDNA of selected strains was amplified by PCR products as described by (Angmo *et al.,* 2016). The PCR primers 27F (5'- TTTGATCCTGGCTCAG-3') and 1492R (5'- GGY-TACCTTGTT ACGACTT-3') were employed during amplification. The DNA sequencing of the PCR product was carried out by Macrogen Sequencing Facilities (Macrogen-Korea, Seoul, and 168 Korea). The sequencing results were aligned with the NCBI database using the BLAST algorithm. Accession numbers were received for selected LAB isolates by GenBank. The neighbor-joining method was applied to determine the closest bacterial species using MEGA software 7.0 (The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4(4), 406-425.

Evaluation of probiotic characteristics

Acid tolerance

The simulation of gastric conditions was carried out as described by (de Almeida Júnior *et al.,* 2015).

The isolates were harvested by centrifugation (3000 × g, 10 min) and washed three times with phosphate buffered saline (PBS; 9g/l NaCl, 9g/l Na2HPO 4.2 H_2O, 1.5g/l KH2PO4, pH 6.2). The cells were resuspended in PBS, the pH was lowered to 2, 3 and 7, and the cells were then incubated at 40°C at each pH for 24 hours under the same conditions. At the end of incubation, the viable cells were enumerated by pour plate counts on M17 agar.

Resistance to bile salts

The resistance of the strains to bile was assessed according to (Malakar *et al.,* 2017). The isolates growing in M17 broth were harvested by centrifugation (10.000 × g for 10 min at 4°C). The tolerance of the strains towards bile salts was evaluated in 10 ml of sterile M17 broth supplemented with a mixture of sodium cholate and sodium deoxycholate (Sigma) in a ratio of 1:1, reaching final concentrations of 0%(control),. 0.3% and 2%. Total viable counts were determined after exposure to bile salts for 24 hours by the pour plate method with M17 after serial dilutions of the sample and incubation at 40°C for 24 hours. The experiment was performed in triplicate.

Hemolytic activity

The strain was tested for hemolytic activity using blood agar (7% v/v sheep blood) and incubating for 48 h at 40°C as described by (Pieniz *et al.,* 2014) . The hemolytic reaction was recorded by the observation of partial hydrolysis of red blood cells. Strains that produced green-hued zones around the colonies (α-hemolysis) or did not produce any effect on the blood plates (γ-hemolysis) were considered nonhemolytic. Strains displaying blood lysis zones around the colonies were classified as hemolytic (β-hemolysis). The experiment was performed in triplicate.

Antibiotic susceptibility

The patterns of resistance or sensitivity to antibiotics of the LAB isolates were determined using the disc diffusion method according to the recommendations of the Clinical and Laboratory Standards Institute (Wayne 2010). LAB strains were incubated overnight under anaerobic conditions at 40°C and 5% CO2. Exactly 100 ml of the diluted cultures (approximately 10^7-10^8 viable cells) were diffused onto Mueller-Hinton agar, and different antibiotic discs containing penicillin G (10 units), colistin (10mg), chloramphenicol (30mg) vancomycin (30mg), tetracycline (30mg) and ciprofloxacin (5µg) (Bioanalyse AST, Turkish) were applied to the surface using an antibiotic disc dispenser. The plates were incubated at 40°C under anaerobic conditions and assessed after 24 h of inoculation. Inhibition zones around the discs were calculated using a digital caliper. The results were expressed in terms of resistance, moderate susceptibility, or susceptibility by comparison with the interpretative zone diameters given by the performance standards for antimicrobial disc susceptibility tests (Abushelaibi *et al.,* 2017).

Antimicrobial activity

The antimicrobial activity of isolated strains was determined as described by (de Almeida Júnior *et al.,* 2015) using four indicators. These indicator microorganisms were suspended in tryptone salt broth in order to standardize to approximately 10^8CFU/ml, equivalent to 0.5 McFarland turbidity. A suspension of each strain tested was applied to the sterile paper disc (5mm) on plate count agar (PCA) plates previously inoculated with a swab soaked in a culture of each indicator bacteria. The plates were incubated in 40°C for 24 h, and the inhibition zone was assessed by measuring bacterial growth around the discs (radius -mm) and considered positive when it was greater than 2mm. The experiment was performed in triplicate.

EPS production by LAB

EPS production by the LAB isolates was tested according to the method described by (de Almeida Júnior *et al.,* 2015) with modifications. Briefly, LAB cultures were grown in conical asks containing 20 mL of MRS broth supplemented with 2% (w/v) glucose at 40°C for 3 days. Bacterial cells were removed by centrifugation at 6000 rpm for 20 min, and two volumes of cold 95% (v/v) ethanol were added to one volume of culture supernatant for EPS precipitation. Precipitates were recovered by filtration under vacuum and dried at 60°C. Their weight was measured to determine the amount of EPS produced. The experiment was performed in triplicate.

Statistical analysis

Data were statistically analyzed with SPSS (version 6.303). One-way analysis of variance was used to study the means ± deviations (SD), and differences were considered to reach statistical significance when $p \leq 0.05$.

Results and discussion

Isolation and physiological properties of isolates

The isolates selected from spontaneously fermented goat milk of the Relizane region in Algeria showing the typical appearance of LAB on M17 medium (small pinpoint colonies) were randomly selected and assayed for physiological properties. The selected isolates were Gram-positive, catalase-negative rod-shaped bacteria. They were mesophilic and showed good growth at NaCl concentrations of 1.5% and 2.5%. All the isolates hydrolyzed arginine and showed diversity in their ability to ferment different sugars.

Identification by 16S rDNA sequencing

Four strains (E1, E2, E3, and E4) out of 20 isolates belonging to *E. faecium* and showing promise probiotic isolates were selected and identified by 16S rRNA gene sequence and PCR amplification of the 16S rRNA gene, resulting in an amplicon of 1200-1400bp (Fig. 1). Alignments were performed using the BLAST algorithm. The identified isolates were designated as probiotic LAB. The GenBank accession numbers for the 16S rRNA gene sequence of isolates are given in Table 1 Molecular phylogeny analysis and phylogenetic tree analysis were performed to identify LAB to the species level based on the 16S rDNA sequences from evolutionary distances by the neighbor-joining method. The phylogenetic tree of the 4 isolates is shown in Fig. 2. Sequence analysis exhibited that 100% of the 16S rDNA clustered with the sequence of *Enterococcus faecium*.

Table 1. LAB isolates identified by 16S rDNA gene sequencing and their Genbank accession.

Isolate codes	Species	NCBI accession no.
E1	*Enterococcus faecium*	NR115764.1
E1	*Enterococcus faecium*	NR114742.1
E1	*Enterococcus faecium*	LC096216.1
E1	*Enterococcus faecium*	NR113903.1

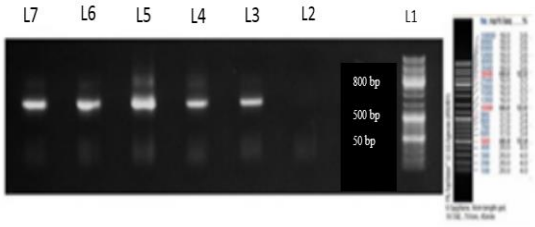

Fig. 1. Polymerase chain reaction (PCR) products obtained from the amplification using the specific 27f/1492R primer. Line 1 = DNA Marker, line 2 = negative control -blank, line 3 =E. faecium isolate (E1), line 4 = E. faecium isolate (E1), line 5 = E. faecium isolate (E1), line6 = E. faecium isolate (E1), line 7 = positive control.

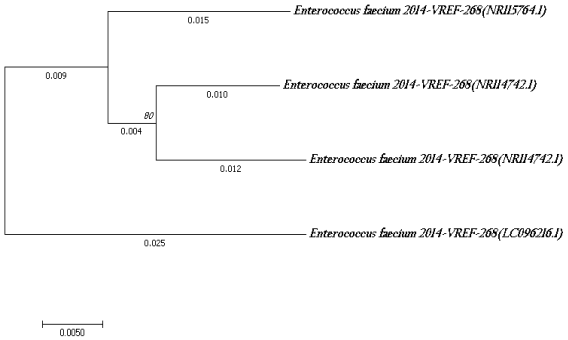

Fig. 2. Neighbor-joining phylogenetic tree based on 16S rDNA sequences. Numbers in parentheses are accession numbers of published sequences.

Probiotic properties

Tolerance to acid and bile salts

To act as a probiotic in the gastrointestinal tract and to exert their beneficial effect on the host bacteria, probiotics must have the ability to survive passage through the stomach and small intestine (Rehaiem *et al.,* 2014) and have good viability in the harsh conditions of gastric and intestinal transit, Therefore, the resistance to low pH and bile salts in the small intestine is one of the important selection criteria for probiotics (Shehata *et al.,* 2016). Thus, the survival of the *E. faecium* strains was first tested under acidic conditions and different bile salt concentrations. Table 2 presents the acid tolerance results of the four *E. faecium* strains at pH 2, 3, and 7 for 24 hours. The strains had counts of 9.07 log CFU/ml at pH 7 after 24 hours and could survive at pH 3 to varying extents.

All the isolates (E1, E3, and E4) except E2 showed a higher level of tolerance in terms of their survival at pH 2 with counts of 7.40 log CFU/ml, 5.00 log CFU/ml, and 6.46 log CFU/ml after 24 hours, respectively. Another important property required in probiotics strains is bile tolerance in order to confirm a high survival rate in the small intestine .The small intestine concentrations vary between 2 and 0.2% (Rehaiem *et al.,* 2014); thus, a level of 0.3% is considered suitable for the selection of resistant strains. The effects of bile salts at concentrations of 0.3% and in 2% on the survivability of the isolated *Enterococcus* species are shown in Table 3. It was found that all the strains (E1, E3, and E4) except E2 showed the highest survivability at 2% bile salts with counts of 5.13 log CFU/ml, 4.47 log CFU/ml, and 4.65 log CFU/ml, respectively. (Malakar *et al.,* 2017) reported the high survivability properties of *Enterococcus* species as potential probiotics in 2% bile salts and observed their tolerance to bile salts at concentrations ranging from 0.3% to 15%. (Rehaiem *et al.,* 2014) revealed that *Enterococcus* species showed considerably stronger tolerance than LAB (e.g., *Lactobacillus* spp.) studied by other researchers. These results indicate the tolerance of the commensal bacteria to the gastrointestinal tract in animals. Overall, the *E. faecium* strains show gastrointestinal transit tolerance and may be used as an alternative bacterium for future probiotic development.

Table 2. Effect of PH on survivability.

Isolate codes	Count in log CFU/ml [a]		
	pH 7	pH 4	pH 2
E1	9.19±0,01	8.96±0,08	7.31±0,04
E2	9.21±0,05	7.42±0,04	5.79±0,72
E3	9.41±0,04	6.45±0,19	00±00
E4	9.10±0,05	7.09±0,04	6.12±0,17

[a] Values represent the mean± s.e.m. of three independent experiments.

Table 3. Effect of bile salts on survivability.

Isolate codes	Count in log CFU/ml [a]		
	0%	0.3%	2%
E1	9.07±0.03	6.12±0.17	5.13±0.32
E2	9.16±0.08	0.00±00	0.00±00
E3	9.02±0.06	5.55±0.13	4.47±0.17
E4	8.73±0.12	5.01±0.06	6.12±0.17

[a] Values represent the mean± s.e.m. of three independent experiments.

Antibiotic susceptibility

The antibiotic susceptibility of isolates is an important characteristics that must be known when screening for the selection of probiotic isolates. The results of the antibiotic susceptibility test are presented in Table 4 and revealed that all the strains of *E. faecium* demonstrated high susceptibility to penicillin G, chloramphenicol, vancomycin, and tetracycline; moderately susceptibility to ciprooxacin; and resistance to colistin. This resistance is due to the absence of the target of this antibiotic in *E. faecium* cells. Thus, the antibiotic susceptibility profile of the *E. faecium* strains is in agreement with previous reports concerning *Enterococcus* strains that are commonly found in foods and have safety criteria. According to the findings of (Malakar *et al.,* 2017), it was possible to use these strains as a probiotics for human and silage inoculants.

Antimicrobial activity

An important step toward the evaluation of probiotics proprieties is determination of antibacterial activity, which is a crucial criterion for potential probiotics from a safety point of view. Table 4 shows the results of the isolates of *E. faecium*, which exhibited antimicrobial activities against different indicator microorganisms, namely, *Listeria monocytogenes* (ATCC 7659), *Escherichia coli* (ATCC 25955), *Staphylococcus aureus* (ATCC 765), and *Salmonella* Typhi (ATCC 25925). The antimicrobial activities of these isolates ranged from very broad spectrum (from 22 to 25mm radius of activity) in the case of *E. coli* to moderately broad spectrum (from 10 to 15mm radius) in the case of *L. monocytogenes* to weak spectrum from (5 to 9mm radius) in the case *Samonella* Typhi. Isolates E4 and E3 exhibited strong antimicrobial activities against 3 of the pathogens but not *Salmonella* Typhi. Interestingly, these isolates showed a greater influence against *E. coli* than they did against the other pathogens (Table 4). The observed antibacterial activity was related to compound such as organic acids and bacteriocins produced by our strains during growth (Chang *et al.,* 2009), and our results were in agreement with the results reported in other works (Barbosa *et al.,* 2014). Additionally, several studies have proved that strains

belonging to the genus *Enterococcus* display many biotechnological properties, such as proteolytic, lipolytic, and esterolytic activity.

Table 4. Antimicrobial activity test.

Isolate codes	Zone of inhibition (mm)[a]			
	S. areus	*E. coli*	*L. Monocytogenes*	*S.* Typhi
E1	17,50±0.2823.56±1.00		10.43±0.29	6.64±0.26
E2	16.66±0.3522.40±0.20		14,20±0.25	8.20±0.15
E3	18.00±0.1125.40±0.66		12.53±0.27	05.26±0.14
E4	19.03±0.1424.46±0.26		15.13±088	9.23±0.08

[a] Values represent the mean± s.e.m. of three independent experiments.

Table 5. Antibiotic susceptibility test.

Isolate codes	Tests of susceptibility					
	Ch	CT	CIP	TE	V	P
E1	27	6	15	29	21	22
E2	20	9	11	32	24	24
E3	26	3	9	31	28	28
E4	24	7	13	34	20	20

(15-20) highly susceptible; (10-14) moderately susceptible; (1-9) susceptible. Susceptibility: zone of inhibition (mm). Ch: chlorampencicol,CT, CT: colistin, CIP: Ciprofloxacin, TE: Tetracycline, P: peniciline G. V : Vancomycin :

Table 6. Hemolytic activity and EPS production.

Isolate codes	Hemolysis	Exopolysaccharides.
E1	γ hemolysis	4mg/l (+)
E1	γ hemolysis	5mg/l (+)
E1	γ hemolysis	3mg/l (+)
E1	γ hemolysis	6mg/l (+)

γ hemolysis: nonhemolytic / (+): weak production.

Conclusions

In conclusion, we used phenotypic and genotypic techniques to confirm that all four strains isolated from Algerian goat milk were *Enterococcus faecium*. Moreover, we proved that these strains have good resistance to gastrointestinal conditions (pH 2, 3, and 7; 0.3% and 2% bile salts) and a good ability to inhibit the growth of many pathogenic microorganisms and are sensitive to a broad range of antibiotics; they also showed nonhemolytic activity. These strains can be considered potential probiotics for inclusion as starter cultures in fermentation processes.

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