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Study of the Antimicrobial and Probiotic Effect of Lactobacillus Plantarum Isolated from Raw Goat's Milk from the Region of Western Algeria

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Abstract

The evolution from a spontaneous fermentation to a directed one is realised with selected lactic starters which give many dairy products processing various organoleptic characters. The integration of new lactic acid bacteria strains isolated from diverse ecosystems is now used to increase the duration of bio-preservation of dairy products. Moreover, some lactic acid bacteria probiotic activity is exploited to produce functional food. The aim of this study is the research of the possible probiotic and technological potential with some preventive and therapeutic characteristics of some Lactobacillus species isolated from Algerian's raw goat's milk. The selected isolate was identify to species level as Lactobacillus plantarum (P6) using API 50CH Kits. Microbiological and biotechnological techniques are used to fulfill this work. Results obtained have shown that Lactobacillus plantarum (P6) can resist to acidic, basic and enzymatic stresses. So the former strain can be considered as a probiotic. Moreover, the inhibition activity of the Lactobacillus plantarum (P6) against pathogens strains (Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25921, Bacillus cereus, Pseudomonas aeruginosa, Vibrio cholerae, Listeria ivanovii ATCC 19119 and Salmonella enterica) is clearly obtained by testing these strains with the direct method. Finely, raw goat's milk can be defined as an ecosystem that promotes the development of a microflora with probiotic characters.

Keywords: Goat's milk; Lactobacillus; antimicrobial activity; probiotic and technologic proprieties; bio-preservation.

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1. Introduction

Lactic acid bacteria (LAB) comprise a wide range of genera and include a considerable number of species. These bacteria are the major component of the starters used in fermentation, especially for dairy products, and some of them are also natural components of the gastrointestinal microflora. Lactobacillus is one of the most important genera of LAB [8]. During the last fifteen years, the Lactobacillus genus has evolved and contains to date more than 80 species. They are present in raw milk and dairy products such as cheeses, yoghurts and fermented milks [8]. Lactobacilli comprise a large and diverse group of gram positive, non- spore forming, catalase negative rod bacteria, able to produce lactic acid as the main end-product of the fermentation of carbohydrates [33,23]. They are considered as generally recognized as safe (GRAS) organisms and can be safely used as probiotics for medical and veterinary applications [13].

Probiotics, as defined in a FAO/WHO (2002) report, are "live microorganisms which when administered in adequate amounts confer a health benefit on the host". Probiotics are beneficial bacteria in that they favorably alter the intestinal microflora balance, inhibit the growth of harmful bacteria, promote good digestion, boost immune function and increase resistance to infection [18].

Other physiological benefits of probiotics include removal of carcinogens, lowering of cholesterol, immunostimulating and allergy lowering effect, synthesis and enhancing the bioavailability of nutrients, alleviation of lactose intolerance [32]. In order to exert their beneficial effect, probiotics must survive in the gastrointestinal (GI) tract, persist in the host, and prove safety for consumer. To survive in the gut, the organisms must be tolerant to low pH and bile toxicity prevalent in the upper digestive tract. Besides, quality assurance programmes associated with research, development, production and validation of the health benefits of these bacteria require their relevant characterization and identification.

Over the world, the research of novel probiotic strains is important in order to satisfy the increasing request of the market and to obtain new functional products. These new functional products must contain probiotic cultures more active and with better probiotic characteristics comparing to those already present on the market.

This work will be focused on the technological, antimicrobial and probiotic properties of Lactobacillus plantarum (P6) isolated from raw goat's milk.

2. *Materi*als and Methods

2.1. Isolation of bacteria:

Twelve samples of raw goat milk were collected from five farms in the western regions of Algeria. Samples were incubated at 37°C until coagulation. Coagulated samples were then activated in MRS broth (De Man et al, 1960) at 37°C for 24h in order to obtain enriched cultures. This culture was streaked on MRS agar medium and incubated [1, 2, 28, 29, 3, 41] under anaerobic condition using a candle extinction jar with a moistened filter paper to provide a CO2-enriched, water-vapor saturated atmosphere at 37°C for 48h. Single colonies picked off the plates were sub cultured in MRS broth at 37°C for 24h before microscopic examination. The cultures of rod-shaped bacteria were streaked on MRS agar medium for purification [6, 28, 3, 29, 4]. Purified strains were stores at −20°C in sterile MRS broth supplemented with 20% glycerol. Additionally, 0.05% cysteine was added to MRS to improve the specificity of this medium for isolation of Lactobacillus [17, 19].

2.2. Preliminary identification of the isolate:

Identification of the isolate at genus level was carried out following the criteria of Sharpe (1979) using morphological, phenotypic and biochemical methods. The cultures were examined microscopically for gram staining and catalase production [15]. In addition, all isolates were tested for growth at 10°C for 10 days, 45°C for 48h and CO2 production from glucose. The pathogenic bacteria (Staphylococcus aureus ATCC 25923, Escherichia

coli ATCC 25921, Bacillus cereus) come from the collection of the microbiology laboratory of the university hospital of Oran.

2.3. Sugar fermentation profile:

The carbohydrate fermentation profile of purified isolate was determined using API 50 CH system (Biomérieux, France). Interpretation of these fermentation profiles were facilitated by systematically comparing all results obtained for the isolates studied with information from the computer-aid database ApiwebTM API 50 CH V5.1 software.

2.4. Simulated GI tract preparation:

Synthesis gastric juices is prepared as described (Glucose 3,5g, NaCl 2,05g, KH2PO4 0,6g, CaCl2 0,11g, KCl 0,37g, Pepsin SIGMA®13,3g, Distilled water s.q.f 1000ml) and adjusted to pH 2 then conserved under freeze conditions [12].

2.5. Bile salttolerance:

The tolerance of lactobacilli to bile salts (BS) was evaluated in MRS supplemented with bile salts using a modified method described by Dora and Glenn (2002). Test lactobacilli isolate culture was grown for 6h in MRS broth at 37°C. An aliquot of 1ml of the 6h old culture was inoculated into 100 ml MRS broth with 0,5% of bile salts (SIGMA®) and adjusted to pH 8.5 [39]. The second contains 0.1% of trypsin (SIGMA®) and α-chymotrypsine (SIGMA®) and adjusted to pH 7 [21, 36].

Bacterial growth was monitored by determination of optical density at 650nm after 6 and 24h incubation period at 37°C. The percent difference between the variation of optical density (DO) of culture without bile salts (ΔDO0% BS) and the variation of optical density of culture containing 0.2 or 0.5% bile salts (ΔDO0.2 or 0.5% BS) would give an index of isolates surviving that can be expressed as follows:

$$Surviving\ (\%) = [(\Delta DO\%\ BS - \Delta DO0.2\ or\ 0.5\%\ BS) / \Delta DO\%\ BS]\ X\ 100$$

Classification criteria included four arbitrary level of bile salt tolerance: excellent if the isolate survived at 0.5% bile salt after 24h; very good if the isolate survived at 0.5% bile salt after 6h but not after 24h; good if the isolate survived at 0.2% bile salt after 24h but not at 0.5% bile salt; 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%.

2.6. Inoculumspreparation:

In order to obtain a rate of concentrations, fresh cultures of Lactobacillus plantarum (P6) are cultivated in MRS media (pH 6.5) and incubated at 37°C. Each two hours OD600 are evaluated to obtain 0.4, 0.5, 0.6, 0.7 and 0.8 of these cultures. Bacterial count of OD600 is obtained by following 1/100 dilutions and successive plating in MRS media pH 6.5 to so we can extrapolate bacterial concentrations on every OD600.

2.7. Resistance to antibiotics:

The antibiotic susceptibility of selected acidotolerant and bile tolerant isolates was determined towards nine antibiotics, namely, penicillin G (6 µg/10 IU), Ampicillin (10µg), Amoxicillin (20µg), Erythromycin (15µg), Tetracycline (30µg), Pristinamycine (15µg), Rifampine (30µg), Cefazoline (30µg) and Ciprofloxacine (5µg). The Strain selection was based on their performance toward acid and bile salts Antibiotic susceptibility was determined semi- quantitatively using a modification of the agar overlay diffusion methods of the National Committee for Clinical Laboratory Standards [30]. Diameters of inhibition zones were measured and results were expressed in terms of resistance (R), intermediate susceptibility (I), and susceptibility (S), according to cut off levels proposed [35, 30, 40].

2.8. Survivalassays:

Fresh cultures of 18hr are harvested and centrifuged at 7000 rpm during 1min, pellets are inoculated in 500µL of synthesis gastric juices; inoculums are incubated at 37°C during 30 min, 1h, 1h30 min and 2h [12, 21]. Following this test, inoculums are centrifuged and pellets are immediately flooded with 500µL of MRS added with 0,5% bile salts and incubated at 37°C during same times [39]. Afterwards, inoculums are once again harvested and auditioned with 500µL of MRS containing 0,1% of trypsin and α-chymotrypsine and incubated at 37°C during same times (30min, 1h, 1h30 and 2h) [21, 36]. The final pellets are added to 500 µL of physiological water and diluted until 10-8, plated on MRS pH 7 and at 37°C during 24h. Colony forming units (cfu) were enumerated after incubation at 37°C during 24 h [25, 5, 24].

2.9. Gelatinaseactivity:

Gelatinase activity of the most antibiotics sensitive isolates was investigated as described by Harrigan and McCance (1990). 2µl of a 6h old culture was spot-inoculated into nutrient gelatin agar. The plates were incubated anaerobically for 48h at 37°C after which they were flooded with saturated ammonium sulfate solution and observed for clear zones surrounding colonies (positive reaction for gelatin hydrolysis). A strain of Staphylococcus aureus ATCC 25923 was used as positive control.

2.10. Haemolysisactivity:

Haemolysis activity of gelatinase negative isolates was investigated as described by [14]. 2µl of a 6h old culture broth was spot- inoculated into sterile blood agar. The blood agar was prepared by adding 7% sheep-blood, that had been preserved in ethylenediaminetetraacetic acid (EDTA), into sterile blood agar base at 45°C. Plates were incubated anaerobically at 37°C for 48h after which they were observed for clear zones surrounding colonies (positive reaction for beta haemolysis). A strain of S. aureus ATCC 25923 was used as positive control.

2.11. Antimicrobialactivity:

Antimicrobial activity of the selected probiotic isolates was checked by using the agar-spot test [24, 25]. Isolates were screened for production of antimicrobial against Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25921, Bacillus cereus, Pseudomonas aeruginosa, Vibrio cholerae, Listeria ivanovii ATCC 19119 and Salmonella enterica as the indicator microorganisms. An aliquot of 2µl of a 6h old producer isolate culture was spotted on MRS agar and plates were incubated anaerobically at 37°C for 48h to allow exhibition of antimicrobial compounds. Cell suspensions of the indicator microorganisms were prepared as follows: each 24h old culture of the indicator strain on Mueller Hinton Agar slant was suspended in sterile physiological saline solution (NaCl 0.9%) and the turbidity was compared to 0.5 Mc Farland (corresponding to108 cfu.ml-1).

50µl of the cell suspension was inoculated in 5ml of Plate Count Soft Agar and overlaid on colonies of producer isolates. After incubation at 37°C for 24h, plates were checked for zones of inhibition surrounding the producer colonies. Inhibition was recorded as positive if the width of the clear zone around the colonies of the producer was 2mm or larger.

The agar well diffusion technique was also used to discriminate antimicrobial activity of the selected probiotic isolates due to organic acid production. The method of [26] was adapted. Isolates were cultured overnight before assay. Bacterial cultures were prepared into cell supernatant pH 7.0. 50µl sterilized free-cell neutralized supernatant was filled into the well against target microorganisms. After 24h of incubation time, the diameter of the inhibition zone was measured and scored. The representation of inhibition zone were not included in 6mm diameter of well. The inhibition zone larger than 2mm was scored positive.

3. Results and discussions

3.1. Choice of effective strain and Preliminary identification:

This specie was chosen because of its capacity to inhibit a broad spectrum of undesirable bacteria. The identification is carried out thanks to the traditional methods established by various authors [22, 6, 19]. The latter is identified to species level by microbiological and biochemical methods, as described by [6]. The strains retained gave small colonies of approximately 1 mm of diameter, lenticular with a white or milky color, smooth surface and a regular circular circumference were observed on solid medium. The microscopic examination revealed that the tested strains were Gram positive, with a cellular rod form associated in pairs or in chains (Table 1).

Table 1. First identification characters of lactobacilli isolates from raw goat's milk

Characters	**P6**
Form	rode
Gram	+
Catalase	-
Fermentation ribose	+
CO2 from glucose	-
CO2 from gluconate	+
Arginine Hydrolysis	-
Growth at 15/45	+/+
	Lactobacillus
Group	Group II, facultative heterofermenter (Streptobacteria)

3.2. Phenotypic identification:

One isolates selected according to their good probiotic potential were identified at phenotypic level as Lactobacillus plantarum (P6) using API 50 CHL technique. This isolate was from samples of various origins. Furthermore, a genotypic method was essential to discriminate between strains. The establishment of the percentage of reliability of this strain in comparison with references Lactobacillus plantarum ATCC 14917 [22, 6].

Microbiological characteristics and fermentation profile of the strain Lb. plantarum (P6) and of the reference strain Lb. plantarum ATCC 14917 are 100% identical, This similarity coefficient is calculated between P6 strain and the reference strain, What guides the identification of the strain (P6) to the species Lactobacillus plantarum.

3.3. Bile salttolerance:

After exposure to acidic conditions, one selected acidotolerant lactobacilli isolates was assayed for bile salt tolerance (Table 2). This isolate demonstrated good capacity to resist bile salts by presenting surviving percentage greater than 50% under exposure to 0.2% bile salts after 24h at 37°C. This isolate was further investigated for their safety properties including sensitivity to antibiotic, haemolysis and gelatinase activity.

Table 2. Surviving percentage of lactobacilli isolates in MRS broth supplemented with 0.2% or 0.5% bile salts after 6h and 24h at 37°C

Isolates	**0,2%**		**0,5%**	
	6h	24H	6h	24h
Lactobacillus plantarum (P6)	90.60	94.55	54.37	88.31
Lactobacillus plantarum (ATCC 14917)	85.31	88.09	51.74	85.43

3.4. Resistance to antibiotics:

One potentially probiotic lactobacilli isolates were subjected to antibiotic susceptibility testing using the agar diffusion method (Table 3). All of them were sensitive to Penicillin, Ampicillin, Amoxicilline, Tetracycline, Pristinamycine, Erythromycin and Rifampine. The isolate Lb. plantarum (P6) demonstrated intermediate resistance to cotrimoxazole. Notable observation is the resistance towards ciprofloxacin expressed by this isolate.

Table 3. Surviving percentage of lactobacilli isolates in MRS broth supplemented with 0.2% or 0.5% bile salts after 6h and 24h at 37°C

Isolates	**Diameter of inhibition zone in mm**								
	P_{10}	**Ap_{10}**	**Am_{10}**	**T_{30}**	**PT_{15}**	**E_{15}**	**RA_{30}**	**C_5**	**CZ_{30}**
Lb. plantarum (P6)	$34_{(S)}$	$31_{(S)}$	$28_{(S)}$	$22_{(S)}$	$23_{(S)}$	$27_{(S)}$	$25_{(S)}$	$0_{(R)}$	$13_{(I)}$
Lactobacillus plantarum (ATCC 14917)	$28_{(S)}$	$33_{(S)}$	$29_{(S)}$	$21_{(S)}$	$20_{(S)}$	$26_{(S)}$	$22_{(S)}$	$0_{(R)}$	$10_{(I)}$

Antibiotics (Disk potency): P10 : Penicillin G (10units) ; Ap10 : Ampicillin (10µg); Am10 : Amoxicilline (10µg); T30: Tetracycline (30µg); PT 15 : Pristinamycine (15µg); E15 : Erythromycin (15µg); RA30 : Rifampine (30µg); C5 :Ciprofloxacine (5µg); CZ30 : Cefazoline (30µg). (S): sensitive; (R): resistant; (I): intermediate.

3.5. Probiotic potential's evaluation:

The results obtained for the aptitude of the strain Lb. plantarum (P6) to resist acidic, basic and enzymatic stresses show clearly that this specie can be regarded as probiotic micro-organism [34]. The concentration at the end of the treatments of Lb. plantarum (P6) is 8.5 log ufc/ml, knowing that according to Salminen et al, 2006 [34], the concentration of administration of a probiotic micro-organism must be 107 or 108 and that according to the definition of FAO/WHO, 2002 [11] probiotic must be administrated with a well-defined concentration so that it can achieve these beneficial effects for its host. In general, a probiotic micro-organism is never administrated in the form of bacterial cream, it is added as an adjuvant in agroalimentary products (Juice, yoghourt… etc) [13, 7] or in the form of capsules containing these freeze-dried micro-organisms. These methods allow, even if the species considered as probiotic resists the gastro-intestinal environment, to increase their chances to arrive in sufficient quantity at the level of intestine [31].

Table 4. Susceptibility of potentially probiotic lactobacilli isolates to antibiotics using the disc diffusion method

		Time			
		30mn	**1h00**	**1h30**	**2h00**
***Lb. plantarum* (P6)**	**OD_{600}**	Growt (Log ufc/ml)	Growt (Log ufc/ml)	Growt (Log ufc/ml)	Growt (Log ufc/ml)
	0,4	8,0	8	7,95	7,88
	0,5	8,1	8,14	8,11	8,07
	0,6	8,3	8,27	8,25	8,2
	0,7	8,43	8,39	8,38	8,36
	0,8	8,5	8,47	8,46	8,44

3.6. Haemolysis and gelatinaseactivity:

The potentially probiotic Lactobacillus isolates was assayed for gelatinase activity and are hemolysis. It showed no activity of gelatinase and positive hemolysis compared to the positive control Staphylococcus aureus ATCC 25923 strains.

3.7. Antimicrobialactivity:

Results for antimicrobial activity of Lb.plantarum (P6) probiotic was as shown in Table 5. Isolate inhibited the growth of all pathogenic strains when agar spot method was used. It was also noticed that, the neutralized free-cell supernatant from the culture of the Lb.plantarum (P6) inhibited the growth of all pathogenic indicators.

Table 5. Inhibitory activity of potentially probiotic lactobacilli isolates

Indicator strains	***Lb. Plantarum (P6)***	***Lb. Plantarum* (P6)(mm)**
Staphylococcus aureusATCC 25923	+	22
Escherichia coliATCC 25922	+	14
Bacillus cereus	+	13
Pseudomonas aeruginosa	+	13
Vibrio cholerae	+	11
Listeria ivanovii ATCC 19119	+	07
Salmonella enterica	+	03

+: Diameter of inhibition zone ≥ 2mm; - : No inhibition; MDR: Multi Drug Resistant.

3.8. Bile salt hydrolase (BSH) activity:

Lactobacillus plantarum (P6) inhibit the growth of pathogens, displayed BSH activity by providing dosage. Lb. plantarum (ATCC 14917) has an activity of average diameter of BSH demonstrating the precipitation zone of 10 mm and Lb. plantarum (P6) shows a strong activity of BSH by expressing the area of diameter greater than 15mm.the zone precipitation around the colonies on the plate.

4. Conclusion:

As a result of research work carried out at the Laboratory of Applied Microbiology, University of Oran, it could be noticed that the strain of Lactobacillus plantarum (P6) can be considered as a new probiotic strain potentially safer with antimicrobial properties. Then will the primer used in the future genotypic study in the agro-food industry and the manufacture of fermented dairy product.

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