Algerian Journal of Research and Technology

ISSN : 2543-3954

A.J.R.T Volume 4 (N° 1) (2020) 25-32

Isolation, identification and study of the probiotic criteria of *Lactobacillus plantarum* isolated from the raw goat milk of the five farms of the Oran region

A. Mami*, M. Djelilate[1], A. Kheloufi[2], A. Mahboubi[3]

**Laboratory of Applied Microbiology, Department of Biology, Faculty of Sciences, University of Oran, Es-senia BP 31100, Oran, Algeria*

[1]Department of Biology, University Center Ahmed Zabana, Bourmadia BP 48000, Relizane, Algeria

[2]Department of Ecology and Environment, Faculty of Natural and Life Sciences, University of Batna-2, Batna BP 05000, Algeria

[3]Department of Living and Environment, Faculty of Natural and Life Sciences, University of Science and Technology Mohamed Boudief of Oran"USTO", El Mnaouar, BP1505, Algeria, 31000 Oran

**Corresponding author: E-mail: anas.mami@yahoo.fr*

Abstract. Lactic acid bacteria (LAB) are widespread microorganisms which can proliferated in all dairy products. The purpose of this study is to provide an evidence of the possible probiotic existence and to access the potential of technology by depicting the main preventive and therapeutic properties of some *Lactobacillus* species. Around 64 strains of *Lactobacillus* were isolated from goat's raw milk of Algeria. The strains which produce antimicrobial substances were detected after their exposure to the solid culture medium. Among all strains, only one strain has exposed an inhibiting activity. This one was identified to a species level by microbiological and biochemical methods. The results achieved so far showed that *Lactobacillus plantarum* can resist to acidic, basic and enzymatic stresses. Indeed, the former strain could be described as a probiotic. Moreover, the inhibition activity of the *Lactobacillus plantarum* against food-altering strains is clearly obtained by testing these strains with direct method. Finely, our results reinforce the hypothesis that the raw goat's milk can provides an excellent ecosystem for the development of a number of micro-flora with probiotic properties.

Keywords. Raw goat's milk, Lactobacillus, Antimicrobial activity, Probiotic, Technologic proprieties, Bio- preservation.

INTRODUCTION

Lactic acid bacteria (LAB) comprise a wide range of genera, including various species. These bacteria are considered as the main catalyzer leading to the fermentation of the dairy products.

In addiction some of them are considered as natural components of the gastrointestinal microflora. *Lactobacillus* is one of the most important genus of LAB (Coeuret et al., 2003). Also, it is generally recognized as safe (GRAS) microorganism. Indeed, *Lacrobacillus* is hugely used as probiotic in medical and veterinary applications (Fuller, 1989).
Probiotic, are nonpathogenic microorganisms with a great health benefit by improving a microbial balance within the host. An extra benefit of probiotic in term of physiology consists on the removal of carcinogens, decreasing of cholesterol's rate, enhancing and synthesizing nutrient's bioavailabilities, alleviation of lactose intolerance immune-stimulating and allergy lowering effect, (Parvez et al., 2006). To be able to survive within gut, the microorganisms need to be more resistant at a low pH condition and bile acid-induced upper digestive tract toxicity. Over the world, the research of novel probiotic strains is important to satisfy various needs of the international market by obtaining new functional products. These new functional products must contain more active probiotic cultures with better features comparing to those exposed in the markets. In this global context, a special attention has been given to study the technological, antimicrobial and probiotic properties of *Lactobacillus plantarum* isolated from raw goat's milk.

MATERIAL AND METHODS

Isolation of bacteria

Twelve samples of raw goat milk were collected from five farms located in Northwestern part of Algeria. Samples were incubated at 37°C until coagulation. Afterwards, coagulated samples were activated in MRS broth (De Man et al, 1960) at 37°C during 24h to obtain enriched cultures. This culture was streaked on MRS agar medium and incubated (Badis, 2004; Badis et al., 2006; Bendimerad et al., 2012; Moulay et al., 2006; Moulay et al., 2013; Zarour et al., 2013) under an anaerobic condition by 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 which have been taken from the plates were sub cultured in MRS broth at 37°C during 24h just before microscopic analyses. The cultures of rod-shaped bacteria were streaked on MRS agar medium for purification (Bendimerad et al., 2012; Benmechernene et al., 2013; Carr et al., 2002; Moulay et al., 2006; Moulay et al., 2013). The purified strains have been stored at −20°C in sterile MRS broth with a supplement of 20% glycerol. Additionally, 0.05% cysteine was added to MRS to improve the specificity of this medium for isolation of *Lactobacillus* (Hartemink et al., 1997; Kihal et al., 2009).

Preliminary identification of the isolate

Identification of the isolate at genus level was carried out according to Sharpe's protocole (1979) by using morphological, phenotypical and biochemical methods. The cultures were examined microscopically for gram staining and catalase production. In addition, all isolates were tested for growth at 10°C first 10 days, and at 45°C for 48h and then CO_2 production from glucose. The pathogenic bacteria (*Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25921, *Bacillus cereus*) have been collected from the laboratory of microbiology at the university hospital of Oran.

Sugar fermentation profile

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

Bile salt tolerance

The tolerance of Lactobacilli to bile salts (BS) had been evaluated in MRS by adding bile salts, (see protocol in Pereira and Gibson, 2002). Tested lactobacilli isolate culture was grown for 6h in MRS broth at 37°C. An aliquot of 1ml of 6h old culture was inoculated into 100 ml MRS broth with 0,5% of bile salts (SIGMA®) and adjusted to pH 8.5 (Shima et al., 2009).
The second culture contains 0.1% of trypsin (SIGMA®) and α-chymotrypsine (SIGMA®) that adjusted to pH 7 (Ruiz-Moyano et al., 2008; Kim et al., 2006).
The growth of studied bacteria was monitored by describing their optical density at 650nm after both 6h and 24h of incubation period at 37°C. The percentage difference between the variation of optical density (OD) of culture without bile salts (ΔOD% BS) and the variation of optical density of culture containing 0.2 or 0.5% bile salts (ΔOD0.2 or 0.5% BS) would give an index of isolates surviving that can be expressed as follows:

$$\text{Surviving } (\%) = \left[\frac{(\Delta OD\%\ BS - \Delta OD0.2 \text{ or } 0.5\%\ BS)}{\Delta OD\%\ BS}\right].100 \qquad (1)$$

Classification criteria may include four level of bile salt tolerance: excellent when the isolate could resist to 0.5% of bile salt after 24h; very good when those isolated could 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 is proved when the percentage of survival is equal or higher than 50%.

Inoculums preparation

In order to obtain the concentration rates, fresh cultures of *Lactobacillus plantarum* 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 extrapolate bacterial concentrations on every OD600.

Survival assays

Fresh cultures of 18h 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, 60 min, 90 min and 120 min (Fernandez et al., 2002; Kim et al., 2006). based on this test, inoculums are centrifuged and pellets are immediately flooded with Five Hundred microlitter (500µL) of MRS added with 0.5% bile salts and incubated at 37°C during the same times (Shima et al., 2009). 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 the same times : 30 min, 60 min, 90 min and 120 min successively (Ruiz-Moyano et al., 2008; Kim et al., 2006). The final pellets were mixed within 500 µL of normal saline and diluted until 10- 8, then plated on MRS at pH 7 and in 37°C during 24h. Colony forming units (CFU) were enumerated after incubation at 37°C during 24 h (Mami et al., 2008; Mami et al., 2012; Boumehira et al., 2011).

Gelatinase activity

Gelatinase activity of the most antibiotics sensitive isolates was investigated as described by Harrigan and McCance (1990). After 6h, two microletter of culture has been 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.

Haemolysis activity

Haemolysis activity of gelatinase negative isolates was investigated according to Gerhardt's et al., 1998 protocole. Two microletter of 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 ethylene-diamine-tetra-acetic acid (EDTA), into sterile blood agar base at 45°C. Plates were incubated in anaerobic conditions at 37°C for 48h, and observed in clear zones that surround colonies (positive reaction for beta haemolysis). A strain of *S. aureus* ATCC 25923 was used as positive control.

Antimicrobial activity

Antimicrobial activity of the selected probiotic isolates was checked by using the agar-spot test (Mami et al., 2012). Isolates were screened for antimicrobial production 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.

A small portion of two microletter of a 6h old isolate culture has been spotted on MRS agar. Plates were incubated at 37°C for 48h within an anaerobic environment to exhibit the 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 to10^8 CFU.ml^{-1}). Fifty microletter of the cell in suspension was inoculated by adding 5 ml of Plate Count Soft Agar and overlaid on colonies of producer isolates.

After 24h of incubation at 37°C, inhibition zones surrounding the producer colonies have been observed in the plates. Inhibition was recorded as positive if the width of the clear zone around the colonies of the producer was 2 mm or larger. The agar well diffusion technique was also used to determine the antimicrobial activity of the selected probiotic isolates caused by organic acid production. Herein, Mant's et al., (2003) protocol had been adapted. Isolates were cultured overnight before assay. Bacterial cultures have been prepared into cell supernatant at pH 7.0. Fifty microletter of a sterilized free-cell neutralized supernatant (NCFS) was filled into the well against target microorganisms. After 24h of incubation time, the diameter of the inhibition zone was measured and scored. The inhibition zone larger than 2mm was scored positive.

RESULTS AND DISCUSSION

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 based on traditional methods (Klein et al., 1998; Kihal et al., 2009). The latter is identified to species level by microbiological and biochemical methods (Carr et al., 2002). 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).

One isolates selected according to their good probiotic potential has been identified at phenotypic level as *Lactobacillus plantar*um using API 50 CHL technique. This isolate was from samples of various origins. Furthermore, a genotypic method was important to discriminate between strains. The establishment of the percentage of reliability of this strain

in comparison with references *Lactobacillus plantarum* ATCC 14917 (Carr et al., 2002; Klein et al., 1998).

Table 1. The first identification features of lactobacilli isolates from raw goat's milk.

Characters	***Lactobacillus***
Form	rode
Gram	+
Catalase	-
Fermentation ribose	+
CO_2 from glucose	-
CO_2 from gluconate	+
Arginine Hydrolysis	-
Growth at 15°C/45°C	+/+
Group	*Lactobacillus* Group II, facultative heterofermenter (Streptobacteria)

Phenotypic identification

Both microbiological properties and fermentation profile of the strain *Lb. plantarum* and of the reference strain *Lb. plantarum* ATCC 14917 are 100% identical. This similarity coefficient is calculated between strain and the reference strain, which help to identify *Lactobacillus* stain to the species of *Lactobacillus plantarum*.

Bile salt tolerance

After exposure to acidic conditions, one selected acidotolerant lactobacilli isolates was assayed for bile salt tolerance (Table 2). This isolate have demonstrated good capacity to resist bile salts by presenting surviving percentage higher 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	95.60	97.55	58.37	**91.51**
***Lactobacillus plantarum* (ATCC 14917)**	**88.31**	**90.09**	**54.85**	**88.49**

Probiotic potential's evaluation

The results obtained for the aptitude of the strain *Lb. plantarum* to resist acidic, basic and enzymatic stresses show clearly that this specie can be regarded as probiotic micro-organism (Perea Velez et al., 2006). The concentration at the end of the treatments of *Lb. plantarum* is 8.5 log CFU/ml, knowing that according to Salminen et al., (2006) (Table 3), the concentration of administration of a probiotic micro-organism must be 10^7 or 10^8 and that according to the definition of FAO/WHO, 2002 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 agro-alimentary products such as Juice and yoghourt (Champagne and Gardner, 2008; Fuller, 1989) 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 get sufficient

quantity at the level of intestine (Pan et al., 2009).

Table 3. The aptitude of the strain *Lb. plantarum* to resist acidic, basic and enzymatic stresses.

		Time			
		30mn	**1h00**	**1h30**	**2h00**
Lb. plantarum	OD600	Growt (Log CFU/ml)	Growt (Log CFU /ml)	Growt (Log CFU /ml)	Growt (Log CFU /ml)
	0.4	8.10	8.14	8.11	7.92
	0.5	8.30	8.27	8,25	8.14
	0.6	8.43	8.39	8.38	8.28
	0.7	8.50	8.47	8.48	8.44
	0.8	8.62	8.56	8.84	8.64

Haemolysis and gelatinase activity

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 of *Staphylococcus aureus* ATCC 25923 strains.

Antimicrobial activity

Antimicrobial activity results of *Lb. plantarum* probiotic were 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* inhibited the growth of all pathogenic indicators.

Table 4. Inhibitory activity of potentially probiotic lactobacilli isolates.

Indicator strains	***Lb. plantarum***	***Lb. plantarum* (mm)**
Staphylococcus aureus ATCC 25923	+	**25**
Escherichia coli ATCC 25922	+	**17**
Bacillus cereus	+	**15**
Pseudomonas aeruginosa	+	**15**
Vibrio cholerae	+	**13**
***Listeria ivanovii* ATCC 19119**	+	**09**
Salmonella enterica	**+**	**06**

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

Bile salt hydrolase (BSH) activity

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

CONCLUSION

The results obtained from one strain of *Lactobacillus plantarum* isolated from the Algerian raw goat's milk may indicate a new probiotic strain potentially safe with antimicrobial properties. Indeed, *Lactobacillus plantarum* strain could have a great impact in the agro-food industry and the manufacture of fermented dairy product. Our lactic acid bacteria are probably the best candidates for improving the microbiological safety of dairy product because they are well

adapted to the various conditions such as the acidic, basic and enzymatic stresses. Our results presented herein suggest that the bacteriocin-producing strain *Lactobacillus plantarum* could be used to improve the safety of the traditional fermented foods of dairy origin.

REFERENCES

Badis A. 2004. PhD thesis. University of Oran.

Badis A., Laouabdia-Sellami N.R., Guetarni D., Kihal M., Ouzrout R., 2006. Sciences Technology. 23. 30-37.

Bendimerad N., Kihal M., Berthier F., 2012. Dairy Sciences of Technology. 92(3), 249-264.

Benmechernene Z., Fernandez I., Kihal M., Böhme K., Calo-Mata P., Velazquez B., 2013. Recent Patents on DNA & Gene Sequences. 7, 66-73.

Boumehira A.Z, Mami A., Henni J.E., Kihal M., 2011. Journal of Pure Applied Microbiology. 5(2), 553-566.

Carr F.J., Chill D., Maida N., 2002. Critique Review of microbiology. 28, 281-370.

Champagne C.P., Gardner N.J., 2008. Food Research International, 1-5.

Coeuret V., Dubernet S., Bernardeau M., Gueguen M.,. Vernoux J.P., 2003. Lait. 83, 269–306.

De Man J.C., Rogosa M., Sharpe M.E., 1960. Journal Applied Bacteriology. 23, 130– 135.

FAO/WHO., 2002. Guidelines for the Evaluation of Probiotics in Food, Report of a joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food, London Otario, Canada.

Fernandez M.F., Boris S., Barbes C., 2002. Journal Applied of Microbiology. 94, 449-455.

Fuller R., 1989. Probiotics in man and animals. Review Journal Applied of Bacteriology. 66, 365-378.

Gerhardt P., Murray R.G.E., Costilow R.N., Nester E.W., Wood W.A., Krieg N.R., Phillips G.B., 1998. American Society of Microbiology,Washington, DC 20006.

Harrigan W.F., Mc Cance M.E., 1990. Academic Press, London.

Hartemink R., Domenech V.R., Rombouts F.M., 1997. Journal of Microbiology Methods. 29, 77–84.

Kihal M., Prevost H., Henni D.E., Benmechernene Z., Diviès C., 2009. Science Research and Essay. 4(11), 1348-1353.

Kim P.I., Jung. M.Y, Chang Y.H., Kim S., Kim S.J., Park Y.H., 2006. Applied Microbiology and Biotechnology. 74, 1103–1111.

Klein G., Pack A., Bonaparte C., Reuter G., 1998. International Journal of Food Microbiology. 41, 103-125.

Mami A., Boumehira A.Z.,. Hamedi A.R., Henni J.E., Kihal M., 2012. African Journal of Microbiology Research. 6(12), 2888-2898.

Mami A., Henni J.E., Kihal M., 2008. World Journal Dairy Food Science. 3, 39-49.

Mante E.S., Sakyi-Dawson E., Amoa-Awua W.K., 2003. International Journal of Food Microbiology. 89, 41-50.

Moulay M., Aggad H., Benmechernene Z., Guessas B., Henni D.E., Kihal M., 2006. World Journal Dairy Food Science. 1, 12-18.

Moulay M., Benlahcen K., Aggad H., Kihal M., 2013. Advances in Environmental Biology. 7(6), 999-1007.

Pan X., Chen F., Wu T., Tang H., Zhao Z., 2009. Food Control. 20, 598-602.

Parvez S., Malik K.A., Ah Kang S., Kim H.Y., 2006. Journal Applied Microbiology. 100, 1171-1185.

Perea Velez M., Hermans K., Verhoeven T.L.A., Lebeer S.E., Vanderleyden J., De Keersmaecker S.C.J., 2006. Journal Applied Microbiology. 1-9.

Pereira I.A.P., Gipson R.G., 2002. Applied Environment Microbiology. 68, 4689-4693.

Ruiz-Moyano S., Martin A., Benito M.J., Nevado F.P., de Guia Cordoba M., 2008. Meat Science. 03, 1-11.
Salminen S., Benno Y., de Vos W., 2006. Journal Clinique Nutrition. 15, 558-562.
Sharpe M.E., 1979. Academic Press, London. 233-259.
Shima M., Matsuo T., Yamashita M., Adachi S., 2009. Food Hydrocolloids. 23, 281-285.
Zarour K., Benmechernene Z., Hadadji M., Moussa-Boudjemaa B., Henni J.E., Kihal M., 2013. Revue «Nature & Technologie». B-Sciences Agronomiques et Biologiques. 08, 39-47.