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Technological Characterization of Lactic Acid Bacteria Isolated from Different Sheep's Milk

L. Ketrouci, F. Dalache, D. Benabdelmoumene[1], A.A. Dahou, A. Homrani

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ABSTRACT

Background: Technological characterization of lactic acid bacteria isolated from sheep's milk collected in 3 regions in northwestern Algeria.

Methods: During the period from 2018 to 2019, fifty strains of Lactic acid bacteria isolated from samples sheep's milk were evaluated for several technologically-relevant properties: diacetyl and exopolysaccharides production, acidification, proteolytic and lipolytic activity and their antagonist activity against *Escherichia coli* and *Pseudomonas aeruginosa*.

Result: The results indicate that among all the isolates only 20% were distinguished by their production of EPS mainly the genus *Leuconostoc*. Diacetyl production was observed in 71% of *Lactobacillus*, 60% of *Enterococcus* and 25% in *Leuconostoc*. 94% isolates showed moderate proteolytic activity. 56% and 60% of the strains degraded tween 80 and olive oil respectively for lipolytic activity. Inhibition activity by the cultures LAB was about 82% and 78% against *E. coli* and *P. aeruginosa* respectively. No culture supernatants inhibit *P. aeruginosa*, however 18% of the *Enterococcus* trains inhibit *E. coli*. BME1.A2 and BME2.D4 showed their highest acidification capacity developing a very large quantity of lactic acid after 24 h of incubation, *i.e*., 7.6 and 8.4 g lactic acid/L respectively.

Key words: Antagonist activity, Lactic acid bacteria, Sheep milk, Technological properties.

INTRODUCTION

Sheep's milk is an interesting raw material because it is rich in nutrients and contains high concentrations of total solids (Balthazar *et al.,* 2017). This product has specific characteristics which make it the noblest milk indeed lipid and protein contents are twice as high as those found in milks of other dairy species (cows and goats).

Lactic acid bacteria (LAB) constitute a highly phylogenetically heterogenous bacterial group, having the GRAS (Generally Recognized as Safe) status because they are nonpathogenic, suitable for technological and industrial processes (Shehata *et al.,* 2016). LAB are ubiquitous in the environment and are considered to be the dominant microbiota in milk and dairy products. Some characteristics of LAB, such as acid production, probiotic, proteolysis, lipolysis and autolysis have contributed to their use as starters, and are investigated when selecting these bacteria. Moreover, exopolysaccharides (EPS) production by LAB received increasing attention due to their immunogenic properties (Cuffia *et al.,* 2020). EPS also contributes to the mouth-feel, texture and taste perception of fermented dairy products (Patil *et al.,* 2015).

MATERIALS AND METHODS

Milk samples, isolation and identification of LAB

50 strains of LAB were isolated from different samples sheep's milk collected in 3 regions of North-Western of Algeria, namely, three samples sheep's milk from Naâma (BNF1, BNF2 and BNF3), two samples from Mecheria (BME1 and BME2) and two samples from Mascara (BMA1 and BMA2).

Sciences and Technics of Animal Production Laboratory, Abdelhamid Ibn Badis University, Mostaganem, Algeria.
[1]Applied Animal Physiology Laboratory, Abdelhamid Ibn Badis University, Mostaganem, Algeria.

Corresponding Author: L. Ketrouci, Department of Agronomic Science, Specialty of Animal Production and Biotechnology, Faculty of Natural and Life Sciences, Abd El-Hamid Ibn Badis University, Mostaganem. Email: leyla.ketrouci@univ-mosta.dz

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All strains were identified using phenotypic tests and only 18 strains were performed using the MALDI-TOF MS Biotyper (Bruker Daltonik GmbH, Bremen, Germany) (Data not shown). All experiments were performed in the Laboratory of Sciences and Technics of Animal Production, Faculty of Nature and Life Sciences, Abdelhamid Ibn Badis University, Mostaganem, Algeria.

Biotechnological characterization

EPS production

Production of EPS was screened in MSE medium (sucrose (10%) after incubation at 30°C for 24 h. EPS production was assessed based on the presence of mucoïd strains and are characterized by the formation of large, slimy and sticky colonies (Fguiri *et al.,* 2016).

Diacetyl production

Diacetyl production was detected by the Voges Proskauer (VP) reaction. Strains were seeded on MRS medium, incubated at 30°C for 24 h. Then,0.5 mL of 1% (wt/vol) α-naphthol solution plus 0.5 mL of 16% (wt/ vol) sodium hydroxide solution was added to 1 mL of microbial culture. After 10 minutes, positive result was visualized by a red ring at the top of the culture (Ribeiro *et al.,* 2014).

Evaluation of proteolytic activity

Proteolytic activity was determined as by De-Almeida *et al*. (2015). LAB strains were spotted on surface of MRS agar supplemented with 10% skimmed milk (wt/vol) and incubated at 30°C for 72 h. Positive result was indicated by a clear zone around the spots.

Evaluation of lipolytic activity

Lipolytic activity was determined on solid MRS medium (at pH 7) supplemented with different lipid substrates. This activity was detected on MRS devoid of Tween 80 and supplemented with 3% olive oil. For artificial source, Tween 80 (3%) was used. Plates were inoculated with the corresponding culture in spots and incubated at 30°C for 72 h. Lipolytic activity was detected by clear zones surrounding the spots (Hantsis-Zacharov and Halpern, 2007).

Acidification activity

200 mL of skim milk (10%) were inoculated (1% v/v) with each strain from 16 selected LAB and incubated at 30°C. Titratable acidity was determined by titrimetric method as described by AOAC (2000) and measured at 0, 2, 4, 6, 8, 10, 12 and 24 h. For most samples, 1% phenolphthalein (1g phenolphthalein in 100 mL ethyl alcohol 95°) was added (0.5 mL) and the sample was titrated with N/9 sodium hydroxide to the first permanent (30 s) color change to pink. pH was measured using a pH meter (PHSJ-3F). Acidity in dairy products is expressed in Dornic degree (1°D = 0.1 g/L of lactic acid).

Antibacterial activity
Agar spot test method

Selected microorganisms were screened using the agar-spot-test method against two pathogen bacteria of test microorganisms *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). LAB strains were spotted on the surface of MRS or BHI containing 1% agar, seeded with one of the test microorganisms (10^8 UFC/mL), and incubated for 24 h at 37°C (Fleming *et al.,* 1975). Results were determined by measuring clear zones of the inhibition.

Agar well diffusion method

Inhibitory activity was determined, according to Harris *et al.* (1989). indicator strains: *E. coli* and *P. aeruginosa* were grown in Tryptone Soya Agar (TSA, Himedia) supplemented with 0.6% Yeast extract for 24 h at 37°C. Each pathogen was suspended in 4 mL of sterile water and standardized to approximately 10^8 CFU/ mL and 1 mL was spread on the surface of plate with MRS agar and allowed to absorb. After, 70 µL of cell free supernatant obtained by centrifugation (Sigma 4-16 KS N°136689) (6000 rpm /10 min at 4°C) from each isolate of LAB in exponential growth phase were placed into wells bored of 5 mm in diameter in agar plates.

After diffusion of supernatants for 18 h at 4°C, plates were incubated for 24 at 37°C. Inhibitory activity was asset by measuring the dimension of the clear halos.

All experiments of technological properties were performed in triplicate.

Statistical studies

The data collected was subjected to an analysis of variance (SAS Institute, 2008). A one-way ANOVA analysis was applied to the results obtained from the activities, using the Student-Newman-Keuls test for comparison of the mean values (P<0.05).

RESULTS AND DISCUSSION

Technological properties

According to phenotypic identification, *Enterococcus* genus was the most group frequently isolated from sheep's milk with a predominance of 70%, followed by *Leuconostoc* with 16% and *Lactobacillus* with 14%. The proportions rate of the different bacterial groups are eminently variable from milk to another, in relation to the variety of operating environments (Dahou *et al.,* 2020).

EPS production

EPS production was observed in only 10 strains isolated mainly from the two milk samples collected in the region of Mascara, BMA1 and BMA2. All strains belonging to the genus *Leuconostoc (Ln)* and 2 strains belonging to the genus *Enterococcus* (*En*) were found to produce EPS with large and sticky colonies (Table 1). Our results corroborate with those described by Maina *et al.* (2008), who showed that several strains of *Leuconostoc* (*Ln. mesenteroïdes* and *Ln. citreum*) are known to produce exopolysaccharides, which are mainly dextran, which is the case of the three species *Ln. mesenteroïdes ssp dextranicum* (BMA1.A5, BMA2.B1 and BMA2.E2). Also, Patil *et al.*, (2015), showed that LAB isolated from different milk's produced EPS and played important role in improving of flavor and texture of fermented dairy products.

Diacetyl production

In our study, 56% of the isolates produced diacetyl (Table 1). 71.42% of the *Lactobacillus* (*Lb*) produce diacetyl. In agreement with our results, Nikolic *et al.* (2008) also observed that a high proportion of *Lactobacillus paracasei* isolated from goat cheeses had the capacity to produce diacetyl. 60% of the isolates of *Enterococcus* produce diacetyl. 3 strains were considered as high-level diacetyl producers, *Lb plantarum* (BNF1.A8) and two strains of *Enterococcus* (BNF2.B3 and BNF3.B5). However, only 25% of the *Leuconostoc* genus strains produce diacetyl. Moreover, Garabal *et al.* (2008) showed that optionally heterofermentative *Lactobacillus* produce the highest

Table 1: Technological activities of LAB isolated from sheep's milk.

LAB strains	Identification	EPS production	Diacetyl production	Proteolytic activity	Lipolytic activity		Antimicrobial activities (Zone of inhibition[a])			
							E. coli[b]		*P. aeruginosa*[c]	
					Olive oil (3%)	Tween 80 (3%)	Spot test	Well diffusion	Spot test	Well diffusion
BNF1.A2	*Lb. paraplantarum*	-	-	+	+	-	++	-	++	-
BNF1.A3	*Lb. plantarum*	-	+	+	+	+	+	-	+	-
BNF1.A5	*Lactobacillus*	-	+	+	-	-	+	-	+	-
BNF1.A8	*Lactobacillus*	-	+	-	+	+	+	-	+	-
BNF2.C5	*Lactobacillus*	-	+	+	+	-	++	+	++	-
BNF2.A6	*Lactobacillus*	-	-	+	+	-	+	-	+	-
BNF2.A8	*Lactobacillus*	-	+	+	+	-	+	-	+	-
BMA1.A2	*Ln. mesenteroïdes ssp mesenteroïdes*	+	-	+	-	+	+	-	+	-
BMA1.A5	*Ln. mesenteroïdes ssp dextranicum*	+	-	+	-	+	-	-	-	-
BMA1.C9	*Leuconostoc*	+	-	+	-	-	+	+	++	-
BMA1.E1	*Leuconostoc*	+	-	+	-	+	+	-	+	-
BMA1.E3	*Leuconostoc*	+	-	+	-	+	+	-	+	-
BMA2.B1	*Ln. mesenteroïdes ssp dextranicum*	+	++	+	-	+	-	-	-	-
BMA2.E1	*Leuconostoc*	+	-	+	-	+	+	+	+	-
BMA2.E2	*Ln. mesenteroïdes ssp dextranicum*	+	+	+	+	+	-	-	-	-
BNF2.A9	*Enterococcus*	-	-	+	+	+	+	-	+	-
BNF2.B3	*Enterococcus*	-	++	-	+	+	++	-	+	-
BNF2.B5	*Enterococcus*	-	-	+	+	+	+	-	+	-
BNF2.b2	*Enterococcus*	-	-	+	+	-	+	-	+	-
BNF2.C5	*Enterococcus*	-	-	+	+	-	+	-	+	-
BNF2.D5	*Enterococcus*	-	+	+	+	+	++	-	+	-
BNF2.D8	*Enterococcus*	-	+	+	+	-	+	-	+	-
BNF2.F1	*Enterococcus*	-	+	+	+	+	+	-	+	-
BNF2.f1	*Enterococcus*	-	+	-	+	+	+	-	+	-
BNF3.A2	*Enterococcus*	-	+	+	+	+	+	-	+	-
BNF3.A3	*Enterococcus*	-	+	+	+	+	++	-	++	-
BNF3.A6	*Enterococcus*	-	-	+	+	+	+	-	+	-
BNF3.B5	*En. durans*	-	++	+	+	+	-	-	-	-

Table 1: Continue....

Table 1: Continue....

BNF3.C3	*Enterococcus*	-	+	+	-	+	-	-	-	-
BNF3.D4	*En. durans*	-	+	+	-	+	+	-	-	-
BNF3.F1	*Enterococcus*	-	+	+	+	+	+	-	+	-
BNF3.G3	*En. durans*	-	+	+	-	+	+++	-	++	-
BMA1.A1	*En. durans*	-	-	+	-	+	++	-	+	-
BMA2.A2	*En. durans*	+	+	+	+	-	-	-	-	-
BMA2.A3	*Enterococcus*	+	+	+	+	+	-	+	-	-
BMA2.C3	*En. durans*	-	-	+	+	-	-	-	+	-
BMA2.D3	*Enterococcus*	-	-	+	-	-	-	+	-	-
BMA2.F2	*En. durans*	-	-	+	-	+	+	+	+	-
BME1.A2	*En. durans*	-	-	+	-	-	+++	-	+	-
BME1.A3	*Enterococcus*	-	-	+	+	+	+	+	-	-
BME1.B5	*Enterococcus*	-	-	+	-	-	+	-	+	-
BME1.E2	*Enterococcus*	-	-	+	-	-	+	+	-	-
BME1.E3	*En durans*	-	-	+	-	-	-	-	-	-
BME2.A1	*Enterococcus*	-	+	+	-	+	++	-	+	-
BME2.A3	*Enterococcus*	-	+	+	+	-	+	-	+	-
BME2.A4	*Enterococcus*	-	+	+	+	-	++	+	+	-
BME2.B2	*Enterococcus*	-	+	+	-	+	+	-	+	-
BME2.C2	*Enterococcus*	-	+	+	+	-	+	-	+	-
BME2.D1	*Enterococcus*	-	+	+	-	-	++	-	+	-
BME2.D4	*Enterococcus*	-	+	+	+	+	++	-	+	-

+ : positive result; - : negative result.

ATCC, American Type Culture Collection; LAB, lactic acid bacteria;

[b]Against *Escherichia coli* ATCC 25922.

[c]Against *Pseudomonas aeruginosa* ATCC 27853.

[a]Results were determined by measuring the diameter of the clear zone in millimeter around the wells.

amounts of diacetyl-acetoin in milk, while *Leuconostoc* produce the lowest amounts of diacetyl-acetoin in acidified milk. This corroborates the results obtained in our study.

Proteolytic activity

Proteolysis is considered to be the most important biochemical traits during cheese maturation. The isolates studied, hydrolyzed the proteins present in the medium with the exception of strains BNF1.A7, BNF2.B3 and BNF2.f1 (Table 1). All *Leuconostocs* were positive for the proteolytic activity. *Lactobacillus* revealed significant proteolytic activity by 6/7 positive results. Our results corroborate with the data reported by Madrau *et al.* (2006) who showed that *Lactobacillus* isolated from traditional Pecorino Sardo cheese and goat's milk had significant proteolytic activity. 33/35 of *Enterococcal* strains are proteolysis positive.

Lipolytic activity

Lipolytic activity with Tween 80 and olive oil, is pronounced in 56% and 60% of the isolates respectively (Table 1). 85% of the *Lactobacillus* strains degrade olive oil and only 28% degrade Tween 80. Nieto-Arribas *et al.* (2010) showed that *Lactobacillus* isolated from Tenerife cheese and Manchego cheese have no lipolytic activity on tributyrin agar. For *Enterococcus* genus, 62% showed positive lipolytic activity with olive oil and 60% with Tween 80. *Enterococcus* are the main genus contributing to the lipolysis of cheese (Giraffa, 2003). For *Leuconostoc* genus, 87% strains degraded tween 80 and only 14% degrade olive oil.

Acidifying power

The results of acidification activity of 16 strains studied (10 *Enterococcus*, 3 *Lactobacillus* and 3 *Leuconostoc*) revealed differences in acidifying power after 24 h of incubation (Fig 1).

LAB strains have their ability to ferment lactose into acid lactic (Saha *et al.,* 2017). For *Enterococcus* genus, all the strains tested gave acidification values between 8.6 g/L (BME2.D4. *En durans*) and 4 g/L (BME2.C2. *Enterococcus*). pH changes by our *Enterococcus* strains ranged between 5.78 and 5.01 of incubation (Fig 1).

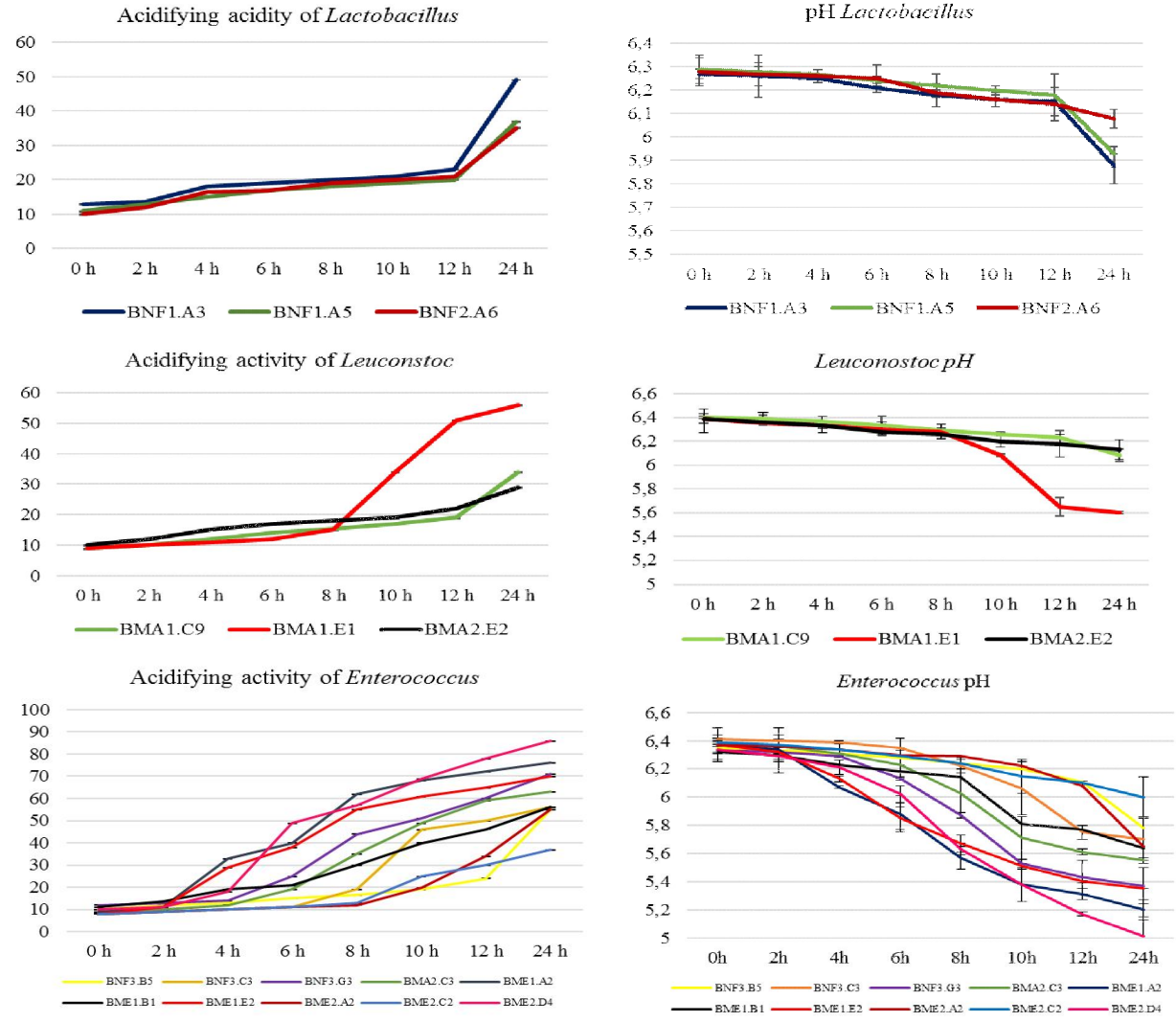

Fig 1: Acidifying activity and pH of 16 strains of lactic acid bacteria isolated from different sheep's milk.

Lactic acid production by strains belonging to *Lactobacillus* strains was low except for the *Lb plantarum* (BNF1.A3) which showed moderate acidifying activity, *i.e*., 4.9 g/L compared to the BNF2.A6 (*Lactobacillus*) strain where the amount of lactic acid produced was 3.5 g/L. *Lactobacillus* strains slowly metabolize lactose (Carafa *et al.,* 2015).

The production of lactic acid by the *Leuconostoc* strains was significantly lower than the other two genus studied. The acidification capacity was of the order of 5.6 g/L (BMA1.E1. *Leuconostoc*) and 2.9 g/L (BMA2.E2 *Ln. mesenteroïdes ssp dextranicum*) and pH values reached with these strains vary between 6.13 and 5.60 after 24 h of incubation.

Antimicrobial activity

We classified the inhibitions observed in our results in 3 types depending on the dimension of the halos in low (+: 1-2 mm) to moderate (++: 2-4 mm) and high (+++: >4 mm) (Table 1). *Lactobacillus* (*Lb plantarum*) and *Enterococcus* (*En. durans*) strains were found to have a higher antagonistic activity against pathogens. The inhibitory effect is at least weakest for *Leuconostoc* strains.

For *Lactobacillus* genus, all strains isolates showed inhibitory activity against both pathogens tested, however no supernatant showed inhibitory activity except *Lactobacillus plantarum* species which showed moderate inhibition against *E. coli*.

Leuconostoc genus had the lowest antimicrobial activity against *E. coli* and *P. aeruginosa* with the exception of the three *Ln. mesenteroïdes ssp dextranicum* species (BMA1.A5, BMA2.B1 and BMA2.E1) which did not inhibit the indicator strains.

Most strains belonging to the *Enterococcus* genus have shown their inhibitory effect against both pathogens. *En. durans* species strongly inhibited *E. coli* with an inhibition zone exceeding 4 mm. No supernatant was able to inhibit *P. aeruginosa*, but 6 strains of *Enterococcus* inhibited *E. coli.* Thus, these data demonstrate that the antagonistic activity shown against indicator pathogens allows the application of the LAB studied as bioconservatives in the production of dairy products, increasing their shelf life.

CONCLUSION

The 50 strains isolated from sheep milk showed efficient technological activities. Our LAB strains have high potential for probiotic application, with elevated production of EPS by *Leuconostoc* genus, high diacetyl production and also lipolytic and proteolytic activity by *Lactobacillus* which confers them to have a potential to be used as adjunct cultures in cheese-making. *Enterococcus* genus showed the most efficient antimicrobial activity against *E. coli* and *P. aeruginosa.*

High variability in technologically revealing traits was found among isolates and could be the basis for the selection of strains specifically to be used as a supplement culture in the production of dairy products and other biotechnological applications.

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