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Lactic acid bacteria from Jijel's traditional butter: Isolation, identification and major technological traits

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RESUMEN

Bacterias acidolácticas de mantequilla tradicional de Jijel: aislamiento, identificación y principales características tecnológicas

Se aíslan veintisiete (27) bacterias acidolácticas de la mantequilla tradicional de Jijel. Éstas pertenecen a los géneros *Lactococcus, Lactobacillus* y *Leuconostoc.* Los resultados muestran que *Lactobacillus plantarum* es la especie predominante en dicha mantequilla. Diversas cepas presentan algunas propiedades tecnológicas interesantes.

PALABRAS CLAVE: Bacterias acidolácticas – Identificación – Mantequilla tradicional – Propiedades tecnológicas.

SUMMARY

Lactic acid bacteria from Jijel's traditional butter: Isolation, identification and major technological traits

Twenty seven (27) lactic acid bacteria were isolated from Jijel's traditional butter. These strains belong to three genera: *Lactococcus, Lactobacillus* and *Leuconostoc*. The results showed that *Lactobacillus plantarum* was the predominant species in this traditional butter. It appears that these strains have some interesting technological properties.

KEY-WORDS: Identification – Lactic acid bacteria – Technological properties – Traditional Butter.

1. INTRODUCTION

Lactic acid bacteria (LAB) occur naturally in several raw materials such as milk, meat and flour (Rodriguez *et al*., 2000). LAB are used as natural or selected starters in food fermentations in which they perform acidification due to the production of lactic and acetic acids. The protection of food from spoilage and pathogenic microorganisms by LAB is related to their antagonistic activity (Parvez *et al.,* 2006).

LAB play an important role in food fermentation as the fermented products are characterized by hygienic safety, storage stability and attractive sensory properties (Savadogo *et al.,* 2006; Kacem *et al.,* 2004).

Interest in microorganisms as a component of biological diversity has been renewed in recent years. The interest in the microorganisms occurring in foods is primarily due to the biotechnological potential of new bacterial species and strains (Leisner *et al.,* 1999). LAB are widely distributed in nature and occur naturally as indigenous microflora in raw milk and these gram positive bacteria also play an important role in many food and feed fermentations. Microorganisms of the genera *Lactococcus*, *Lactobacillus, Leuconostoc*, *Streptococcus* and *Pediococcus* are involved in several fermentations (Soomro and Kiran, 2002).

Fermented dairy products are an important integral part of the diet in Algeria. The major traditional butter produced in Jijel is called "Dhan". Rural people still produce "dhan" by traditional methods using primitive utensils. The raw material is cows' milk, after spontaneous coagulation it is churned and then the butter is recovered.

To our knowledge there is still no information about the lactic acid bacteria of this traditional butter.

The aim of this study is the isolation and identification of lactic acid bacteria from this traditional butter.

2. MATERIALS AND METHODS

2.1. Butter samples

The butter used in this study was the traditional "dhan" made from raw cows' milk. Five samples of butter were collected from local retailers in the region of Sayda-Zguiwartan, south of Jijel in Algeria.

2.2. Isolation of bacterial strains

Samples were heated at 45 °C and then centrifuged at 3000 rpm for 15min. The intermediate or central liquid phase was recovered and then five decimal dilutions were carried out (Leveau and Bourgeois, 1980). Dilutions of 10^{-4} and 10^{-5} were plated in duplicate onto MRS agar and M17 agar. The plates were incubated at 37 °C for 24 h in anaerobic conditions.

Twenty seven (27) colonies were picked from the higher dilutions (10^{-5}) and sub-cultured in MRS and M17 broth.

2.3. Physiological and biochemical tests

The identification of the isolates was performed according to the criteria of Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 1994) and using the methods and criteria of (Sharpe 1979).

The isolates were initially subjected to the Gram staining and the catalase test (3 % H_2O_2). Only the Gram positive, catalase negative isolates were further identified. Growth at different temperatures was determined in MRS and M17 broths at 10 °C, 15 °C, 40 °C and 45 °C. NaCl tolerance (4 % and 6.5 %) was performed in MRS and M17 broths; reductase, hydrolysis of arginine and Sherman tests (milk with 0.1 % and 0.3 % of methylene blue) were also recorded. The acetoin production was determinate using the Voges-Proskauer test.

The fermentative type was determined on agar (Gibson and Abdelmalek, 1945). This agar was prepared and autoclaved in accordance with the manufacturer's instructions. Citrate utilization and haemolysis type were also determined.

The ability of the isolated strains to produce acid from different carbohydrates was determined by API 50 CHL/CHS test kits (BioMerieux, S.A., France). The API test strips were prepared as recommended by the kit supplier and scored after incubation for 24 and 48 hours at 37 °C. The results were loaded onto the API system software, which used the phenotypic data to predict a species identity (%) for each isolate.

2.4. Assessment of technological performance of some isolated strains

The first technological aptitude of the studied isolated strains was evaluated according the terms of the acidification rate and coagulation of skim milk. The determination of the total acidity (°D) was performed by titration with N/9 NaOH in the presence of phenolphtaleine.

The exopolysaccharide production from sucrose was recorded in MRS agar (20 $g.L^{-1}$ sucrose) for lactobacilli and in M17 agar for *Lactococcus* and *Leuconostoc* (Leveau *et al.,* 1991).

The proteolytic activity was evaluated in Yeast Milk Agar (YMA). The diameter of proteolysis zone was determined after incubation at 30 °C for 24 hours (Vuillemard *et al.,* 1986).

Some isolates were examined for their positive interactions (cooperation) and negative interactions (inhibition) by the direct method according to (Fleming *et al.,* 1975). Isolates to be tested were spotted onto the surface of MRS agar or M17 agar (10^6 - 10^7cfu/ml from 18-hour cultures in MRS or M17 broth) and incubated overnight at 35 °C. Aliquots (0, 2 ml of 10^5 - 10^6cfu/ml from 12-hour cultures in MRS or M17 broth) of broth culture were inoculated into soft MRS (or M17) agar (7ml) and poured over the plates on which the putative producer had grown. The plates were incubated at 35 °C for 24 hours. Inhibition was scored positive if the width of the clear zone around the colonies of the producer strain was 1.5 mm or larger.

3. RESULTS

3.1. Classification of the isolates

After a series of purification on MRS agar, twenty-three isolates were found to be Gram-positive, catalase-negative, non motile, mesophilic homofermentative bacilli (Table 1). After a series of purification on Elliker medium, four isolates were found to be not motile, Gram positive cocci. These four isolates are able to grow at 10 °C but not at 45°C, while three of them perform gamma haemolysis because the medium is not modified (while *Streptococcus* and *Lactococcus* are gamma haemolytic) and are heterofermentative (Table 2).

The standard physiological and biochemical tests led to identification of the isolates as follows: twenty isolates of *Lactobacillus plantarum* (74.07 %), three isolates of *Lactobacillus curvatus* (11.11 %), one isolate of *Lactococcus lactis*

ssp *cremoris* (3.70 %), one isolate of *Leuconostoc lactis* (3.70 %) and two isolates of *Leuconostoc mesenteroides* ssp *dextranicum* (7.40 %).

The results indicate that members of the same species (*L. plantarum*) have some different physiological and biochemical characteristics and then the percentage of identification using API strip system software is different too. According to our results, *L. plantarum* BJ0022 is able to use inuline, methyl D-mannose and D-raffinose and the percentage of identification using API strip system software is 90.9 % while *L. plantarum* BJ0021 cannot use these three carbohydrates and the percentage of identification is 99.9 %.

Lactobacillus plantarum was the predominant species in the traditional butter (74.07 %), although *Lactobacillus curvatus, Lactococcus lactis* ssp *cremoris, Leuconostoc lactis* and *Leuconostoc mesenteroides* ssp *dextranicum* were present too.

3.2. Acid production

The assessment of the main technological aptitude of some isolated strains was based on the speed of acidification. The time for milk coagulation was used to classify the isolated strains as fast, intermediary and slow acidifying strains. The results showed that *Lactobacillus curvatus* BJ023 and *Lactobacillus plantarum* BJ051and BJ0021 are the fastest strains. *L. curvatus* BJ023 produced 113.33 °D at the end of 12 hours incubation. These three strains were able to coagulate the milk in less than nine hours. Hence, these isolates were the Fast Acid Producers (FAP). On the other hand, *Leuconostoc mesenteroides* ssp *dextranicum* BJ034 was the slowest acidification agent (Figure 1a and Figure 1b)

3.3. Proteolytic activity

The isolates were able to grow on the YMA media where bacterial proteolytic activity led to clear zones. As shown in Table 3, the diameters of

Tabla 1
Physiological and biochemical characteristics of bacilli isolates

Isolates	BJ021	BJ022	BJ033	BJ036	BJ041	BJ045	BJ051	BJ052	BJ0021	BJ0022	BJ0024
Gram Strain	+	+	+	+	+	+	+	+	+	+	+
Cell Shape	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Catalase test	–	–	–	–	–	–	–	–	–	–	–
Growth at:											
15°C	+	+	+	+	+	+	+	+	+	+	+
45°C	–	–	–	–	–	–	–	–	–	–	–
6.5% NaCl	–	–	–	–	–	–	–	–	–	–	–
Arginine hydrolysis	+	+	+	+/–	+	+	+/–	+	+	+	+/–
Fermentative type	h	h	h	h	h	h	h	h	h	h	h
Fermentation of:											
Lactose	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+
Gluconate	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–
Ribose	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–
Xylose	–	–	–	–	–	–	–	–	–	–	–
Identification using API strip system software	96.9%	99.6%	99.9%	99.8%	99.6%	99.9%	99.9%	97.8%	99.9%	90.9%	99.9
Identified as	*Lactobacillus plantarum*										

h: homofermentative.

Tabla 1 (continued)
Physiological and biochemical characteristics of bacilli isolates

Isolates	BJ0031	BJ0032	BJ0034	BJ0041	BJ0042	BJ0331	BJ0341	BJ0411	BJ023	BJ044	BJ0231
Gram Strain	+	+	+	+	+	+	+	+	+	+	+
Cell Shape	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Catalase test	–	–	–	–	–	–	–	–	–	–	–
Growth:											
15°C	+	+	+	+	+	+	+	+	+	+	+
45°C	–	–	–	–	–	–	–	–	–	–	–
6.5% NaCl	–	–	–	–	–	–	–	–	–	–	–
Arginine hydrolysis	+/–	+	+	+/–	+/–	+	+/–	+	+/–	+/–	+/–
Fermentative type	h	h	h	h	h	h	h	h	h	h	h
Fermentation of:											
Lactose	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+
Gluconate	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–
Ribose	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–	+/–
Xylose	–	–	–	–	–	–	–	–	–	–	–
Identification using API strip system software.	99.9%	93.7%	96.9%	99.9%	99.9%	99.6%	94.9%	99.9%	90.5%	99.9%	93.9%
Identified as	*Lactobacillus plantarum*								*Lactobacillus curvatus*		

h: homofermentative

Tabla 2
Physiological and biochemical characteristics of cocci isolates

Isolates	BJ043	BJ032	BJ034	BJ0044
Gram Strain	+	+	+	+
Cell Shape	Cocci	Cocci	Cocci	Cocci
Catalase test	–	–	–	–
Growth:				
10°C	+	+	+	+
40°C	+	–	–	–
45°C	–	–	–	–
4% NaCl	+	n	n	n
6.5% NaCl	–	–	–	–
Sherman test				
0.1% methylene blue	+	n	n	n
0.3% methylene blue	+	n	n	n
Arginine hydrolysis	+	+	+	+/-
Fermentative type	h	het	het	het
Acetoin	–	n	n	n
Haemolysis type	Gamma	Gamma	Gamma	Gamma
Reductase	+	nd	nd	nd
Exopolysaccharides production	nd	–	+	+
Identification using API strip system software	90.1%	92.3%	98.5%	90.8%
Identified as	*Lactococcus lactis* ssp *cremoris*	*Leuconostoc lactis*	*Leuconostoc mesenteroides* ssp *dextranicum*	

h: homofermentative ; het: heterofermentative ; n: not determined.

the hydrolysis zones were from 0 to 6 mm. *Leuconostoc lactis* BJ032 showed the highest score (6 mm) while *Leuconostoc mesenteroïdes* ssp *dextranicum* BJ0044 showed no proteolytic activity. *L. plantarum* and *L. curvatus* BJ023 showed the same scores of protein hydrolysis.

3.4. Exopolysoccharides production

Many dairy LAB are able to produce exopolysaccharides (EPS). The strains producing EPS play beneficial roles in the rheological behaviour and texture of the fermented milks. After incubation, all colonies displayed the characteristics

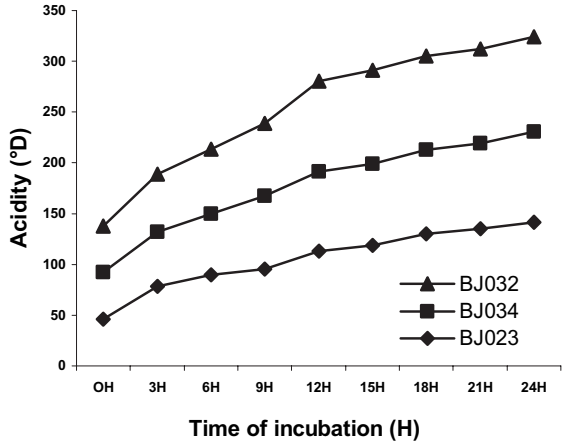

Figure 1a
Production of lactic acid by *L.curvatus* BJ023, *Ln.mesenteroides* ssp.*dextranicum* BJ034 and *Ln.lactis* BJ032.

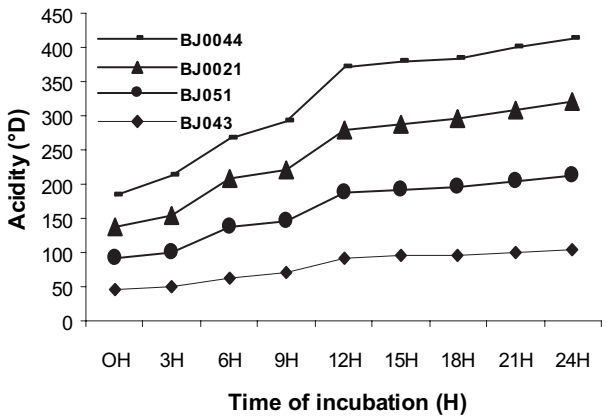

Figure 1b
Production of lactic acid by *Ln.mesenteroides* ssp.*dextranicum* BJ0044, *L.plantarum* BJ0021, *L.plantarum* BJ051 and *Ln.lactis* ssp.*cremoris* BJ043.

of LAB except colonies of *Leuconostoc mesenteroïdes* ssp *dextranicum* BJ034 and BJ0044. The colonies were clearly distinguished (large and pinks colonies) according to their different morphological appearance upon growth in hypersaccharose agar. This could be due to the production of exopolysoccharides.

3.5. Bacterial interaction

The ability to produce pure strains should allow for associating them according to technological criteria, or better yet, according to the quality of the mixture. In both cases, the knowledge of interactions between the strains would be beneficial.

Bacterial interactions were recorded and results indicate that some strains of LAB were able to inhibit the growth of others strains as shown in Table 4. Inhibited strains were called indicator isolates. Among all strains, *Lactobacillus curvatus* BJ023, *Lactococcus lactis* ssp *cremoris* BJ043, *Leuconostoc mesenteroides* ssp *dextranicum* BJ034 and *Lactobacillus plantarum* BJ0021 were able to inhibit respectively 6, 5, 5 and 5 out of 8 indicator isolates.

Table 3
Proteolytic activity of some lactic acid bacteria

Isolates	Proteolytic activity	Diameter of proteolysis zone (mm)
Leuconostoc mesenteroïdes ssp *dextranicum*BJ034	+	5
Leuconostoc mesenteroïdes ssp *dextranicum*BJ0044	–	0
Leuconostoc lactis BJ032	+	6
Lactococcus lactis ssp *cremoris* BJ043	+	5,5
Lactobacillus curvatus BJ023	+	5
Lactobacillus plantarum BJ0021	+	5
Lactobacillus plantarum BJ052	+	5
Lactobacillus plantarum BJ041	+	5

(+): Proteolytic activity positive. (–): Proteolytic activity negative.

Table 4
Interactions between some lactic acid bacteria isolates

Strains	*Ln. mesenteroïdes* ssp. *dextranicum* BJ034	*Ln. mesenteroïdes* ssp. *dextranicum* BJ0044	*Ln. lactis* BJ032	*Lc. lactis* ssp. *cremoris* BJ043	*L. curvatus* BJ023	*L. plantarum* BJ0021	*L. plantarum* BJ041	*L. plantarum* BJ052
Ln. mesenteroïdes ssp. *dextranicum* BJ034	+	+	+	+	+	+	+	+
Ln. mesenteroïdes ssp. *dextranicum* BJ0044	–	+	–	+	+	–	–	–
Ln. lactis BJ032	+	+	+	+	+	+	–	–
Lc. lactis ssp. *cremoris* BJ043	–	–	–	+	–	–	–	–
L. curvatus BJ023	+	+	+	+	+	+	+	+
L. plantarum BJ0021	+	+	+	+	+	–	+	+
L. plantarum BJ041	+	–	–	–	+	+	–	+
L. plantarum BJ052	+	–	–	–	+	+	+	+

(+): Diameter of inhibition > 1.5 mm. (–): Diameter of inhibition = 0 mm.

4. DISCUSSION

From our results, it is clear that lactic acid bacteria were isolated from Jijel's traditional butter. Five LAB species were found which were represented by *Lactobacillus plantarum*, *Lactobacillus curvatus Lactococcus lactis* ssp *cremoris*, *Leuconostoc lactis* and *Leuconostoc mesenteroides* ssp *dextranicum.* These results are not in accordance with various reports indicating that predominant lactic acid bacteria in butter was represented by *Lactococcus lactis* ssp. *cremoris* and *Lactococcus lactis* ssp. *diacetylactis* (Guiraud, 1998).

These results are also not in complete agreement with those reported by (Sagdic *et al.,* 2002) who isolated lactic acid bacteria from traditional Turkish yayik butter represented essentially by strains of *Ln. pseudomesenteroides*, *Ln. gelidum*, *L. delbrueckii* ssp. *bulgaricus* and *E. faecium.*

Lactobacillus plantarum was found in the five butter samples. This strain represented 74.07% of the isolates, *L. plantarum* as well as the other lactobacilli frequently reported to be the dominant microorganisms among the non-starter LAB in long ripening butters or cheeses due to their unique ability to grow in highly hostile environments.

The acidification aptitude (Dornic acidity) of some isolates confirms the weak production of lactic acid. *Lactobacillus curvatus* BJ023 and *Lactobacillus plantarum* BJ051, BJ0021 are the fastest isolates. Our results are not in accordance with those reported by (Zambou-Ngoufak *et al*., 2004) who indicated that *Lactobacillus plantarum* 162RM has very slow acidification properties (milk coagulation was not obtained after 24 hours of incubation). Contrary results were reported by (Haddadin, 2005) who showed that *L. plantarum* and *L. casei* were the fastest isolated strains.

In addition, 87.5% of isolates (7 out of 8 of isolates) showed good proteolytic activity and the highest activity were recorded for *Leuconostoc lactis* BJ032. (Peterson *et al*., 1990) reported that important differences exist among species of LAB in terms of types and quantities of peptidase activities.

Two strains, *Leuconostoc mesenteroides ssp dextranicum* BJ034 and BJ0044, showed their ability to produce exopolysaccharides on M17 media containing 20 $g.L^{-1}$ sucrose. These species have interesting textural technological aptitudes; this type of result was also described by (Colman and Ball 1984). In a previous study, (Bouzar *et al.,* 1996) reported that *L. delbrueckii* ssp. *bulgaricus* CNRZ 1187 and two colonial variants produced different yields of neutral heteropolysaccharides when grown in milk. *L. helveticus* TY 1-2 produced also exocellular polysaccharide (Yamamoto *et al.,* 1994). The presence of viscous extracellular polysaccharides produced by some culture bacteria was also shown to play an important role in achieving satisfactory firmness and apparent viscosity in yoghurt (Patricia *et al.,* 2002).

Finally we found that our lactic acid bacteria strains produce substances that inhibit the growth of other LAB. These results were in accordance with those reported by (Brink *et al.,* 1994) who indicated that LAB exert strong antagonistic activity against many microorganisms, including food spoilage organisms and pathogens. (Parente and Ricciardi 1999) reported that the ability of lactic acid bacteria to compete and finally dominate in fermented products has been attributed to their inhibitory activity due to a decrease in pH, competition for substrates and/or to a variety of antimicrobial agents. If antagonistic interactions between LAB do not depend on bacteriophages, they are caused by liberation molecules like hydrogen peroxide, organic acids or bacteriocins (Aktypis and Kalantzopoulos, 2003).

5. CONCLUSION

Results from this study show the heterogeneity of the lactic acid bacteria in the traditional butter. So, twenty seven isolates were identified; lactobacilli were the predominant (85.19%) in this product. In our study, characterization and identification were based on physiological – biochemical properties and API strip system software analysis. This method is very useful and remains the most widely recognized approach but the classification of isolates would be confirmed by molecular biology techniques such as 16S RNA studies.

The results of the assessment of the technological aptitude indicate that the acidification kinetic confirm the weak production of lactic acid by some isolates; one showed a good proteolysis activity and solely isolates *Leuconostoc mesenteroides* ssp. *dextranicum* BJ034 and BJ0044 showed their ability to produce exopolysaccharide. Also, our lactic acid bacteria isolated from traditional butter exhibited antagonistic activity over other gram positive isolates. For future applications, we would select the best combinations between strains for industrial use.

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