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Lactic acid bacteria from "Sheep's Dhan", a traditional butter from sheep's milk:Isolation, identification and major technological traits

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RESUMEN

Bacterias lácticas de "Sheep's Dhan", una mantequilla tradicional: aislamiento, identificación y principales aspectos tecnológicos.

Veintiséis bacterias lácticas fueron aisladas de "Sheep´s Dhan", una mantequilla tradicional hecha con leche de oveja en Jijel (al Este de Argelia). Estas cepas pertenecen a tres géneros: *Lactococcus, Leuconostoc* y *Lactobacillus*. Los resultados mostraron que *Lactococcus lactis* ssp *diacetylactis* fue la especie predominante en esta mantequilla tradicional. Los resultados de la evaluación de la aptitud tecnológica indican que la principal cepa tiene una buena aptitud de acidificación, algunas de ellas mostraron una buena actividad proteolítica y únicamente *Leuconostoc mesenteroides* ssp. *dextranicum* fue capaz de producir exopolisacárido.

PALABRAS-CAVE: Aspectos tecnológicos – Bacteria láctica – Identificación – Sheep's Dhan.

SUMMARY

Lactic acid bacteria from "Sheep's Dhan", a traditional butter: Isolation, identification and major technological traits.

Twenty six lactic acid bacteria were isolated from sheep's Dhan, a traditional butter made from sheep's milk in Jijel (East of Algeria). These strains belong to three genera: *Lactococcus, Leuconostoc* and *Lactobacillus*. The results showed that *Lactococcus lactis* ssp *diacetylactis* was the predominant species in this traditional butter. The results of the assessment of the technological aptitude indicate that a major strain has a good acidification aptitude, some of them show good proteolytic activity and only *Leuconostoc mesenteroides* ssp. *dextranicum* isolates were able to produce exopolysaccharide.

KEY-WORDS: Identification – Lactic acid bacteria – Technological traits – Sheep's Dhan.

1. INTRODUCTION

Milk fat is one of the major products in the dairy industry and has been primarily used in the production of butter (Arul *et al*., 1988). It is a complex fatty acid composition that results in a unique mixture of triglycerides with a wide range of molecular weights and degrees of saturation (deMan and deMan, 1983).Besides fats, butter contains small percentages of protein, milk sugar and water which make it a suitable substrate for microorganisms (Catsberg and Kempen-van Dommelen, 1990).

The microbiological properties of butter made from different milks have been extensively studied by many researchers (Ubach, 1986; O'Connor *et al*., 1993 and Zhao *et al*., 2000). Some researchers reported that lactic acid bacteria (LAB) (lactococci, lactobacilli, enterococci, streptococci and leuconostocs) were isolated from natural butters (Karahan, 1992 and Sagdic *et al*., 2002). Beerens and Luquet (1987) have reported that *Lactococcus lactis* ssp *diacetylactis* and *Lactococcus lactis* ssp *cremoris* were the predominant species in natural butter.

In Algeria, many studies have been carried out to isolate lactic acid bacteria from cow, sheep and goat's milk (Karam and Zadi, 1994; Karam, 1995; Kacem *et al*., 2003 and Idoui, 2008) or from a traditional butter made from camel's milk called Shmen (Idoui, 1999; Kacem and Karam, 2006; Kacem, 2007 and Idoui et Karam, 2008).

In many regions of East Algeria, the traditional butter is called "Sman", "Dhan" or "Zabda". 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 cow's milk, goat's milk and sheep's milk which, after spontaneous coagulation, is churned, and finally the "Dhan", "Dhan El Maaza" and "Dhan El Naadja" is, respectively, recovered.

To our knowledge there is still no information about the lactic acid bacteria of sheep's Dhan or Dhan El Naadja. The aim of this study was the isolation and identification of lactic acid bacteria from this traditional product and, then to determine its technological properties.

2. MATERIALS AND METHODS

2.1. Sheep Dhan samples

The butter used in this study was the traditional "dhan" made from raw sheeps' milk (Dhan El

Naadja). In the traditional stages of the manufacturing of Dhan, raw sheep's milk is left to sour spontaneously at ambient temperature until coagulation occurs. On gelation, the product is called rayeb; however, by churning the fermentate, the product is separated into lben and raw butter called Dhan. Three samples of butter were collected from local retailers in the regions of Sayda-Zguiwartan and Beni Yadjis south of Jijel in East Algeria. The samples were aseptically placed into sterilized bottles.

2.2. Isolation of bacterial strains

Samples were heated at 45 °C and then centrifuged at 3000 rpm for 15min.The intermediate liquid phase was recovered and then six decimal dilutions were carried out (Leveau and Bourgeois, 1980). Dilutions of 10^{-5} and 10^{-6} were plated in duplicate onto MRS agar (de Man *et al*., 1960) and M17 agar (Terzaghi and Sandine, 1975). The plates were incubated at 32 °C for 24 h in anaerobic conditions.

Twenty-six colonies were picked out from the higher dilutions (10^{-6}) 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) and Kimoto *et al*., (2004).

The isolates were initially subjected to the Gram staining and the catalase tests (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 on MRS and M17 broths; reductase, the hydrolysis of arginine and Sherman tests (Milk with 0.1% and 0.3% of methylene blue) were also recorded. 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 for agar (Solution A: glucose 110g, skim milk 1600 ml; Solution B: peptone 4g, meat extract 8g, NaCl 2g, yeast extract 5.6g, distillate water 400ml, agar-agar 8g. Solution C: tomato juice 200ml, Yeast extract 5.6g. pH7, mix the solutions A, B, C and sterilize). The utilization of citrate was determined in a Kempler and Mc Kay (1980) medium. Haemolysis type was determined in an agar medium with horse blood added (g/l, meat extract 3, peptone 10, NaCl 5, agar-agar 15, sterilized in an autoclave at 120 °C for 15min) (Leveau *et al*., 1991).

The ability of the isolated strains to produce acid from different carbohydrates was determined by API 50 CHL/CHS test kits (Bio Merieux, S.A., S.N 41.0014 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 acidifying property was performed on skim milk. Skim milk powder was weighed and dissolved in water to reconstitute 12% skim milk (w/v), then sterilized using an autoclave at 120 °C for 10min and then cooled at 37 °C. Sterilized milk was inoculated with active culture (1%) of each strain and incubated at 37 °C for 3h, 6h, 12h, 18h and 24 hours.

The determination of the total acidity (°D) was performed by titration with N/9 NaOH in the presence of phenolphtaleine (Accolas et al., 1977). Total acidity (°D) was determined using the following formula: Total Acidity (°D) $= V_{NaOH} \times 10$, Where V_{NaOH} is the NaOH volume used for titration lactic acid contained in 10 ml of fermented milk.

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*. After incubation for 16 to 24 h, mucoid colony formation on agar medium is related to exopolysaccharide production (Leveau *et al*., 1991).

The proteolytic activity was evaluated in Yeast Milk Agar (YMA (g/L): peptone 5, yeast extract 3, non-fat skim milk 1, agar-agar 15, pH7.1, sterilized in an autoclave at 120 °C for 20min). The diameter of proteolysis zone was determined after incubation at 30 °C for 24 hours (Vuillemard *et al*., 1986).

3. RESULTS AND DISCUSSION

3.1. Classification of the isolates

Twenty-six strains were isolated, purified and further identified from traditional dhan made from sheep's milk. All of the isolates were found to be Gram-positive, catalase-negative, non motile and mesophilic (Tables 1, 2 and 3).

Two isolates obtained from MRS agar were homofermentative bacilli, able to grow at 15 °C but not at 45 °C (Table 1). On the basis of physiological and biochemical tests, melibiose fermentation and arginine hydrolysis (Montel *et al*., 1991), these isolates were identified as *Lactobacillus plantarum.*

After a series of purification steps on M17 medium, thirteen isolates were found to be homofermentative cocci, producing gamma haemolysis, reductase positive and promoted growth in a Sherman medium (Table 2). Seven out of 13 of these isolates showed positive acetoin production. The standard physiological and

Table 1
Physiological and biochemical characteristics of *Lactobacillus* isolates

Isolates	Gram	Cell Shape	Catalase test	Growth: 15 °C	Growth: 45 °C	NaCl 4.0%	NaCl 6.5%	Arginine hydrolysis	Fermentative type	Lactose	Sucrose	Gluconate	Ribose	Xylose
SB16	+	Rods	+	+	−	+	−	+/−	h	+	+	+/−	+	−
SB2	+	Rods	+	+	−	+	−	+	h	+	+	+/−	+/−	−
Identified as *Lactobacillus plantarum*														

h: homofermentative; SB: sheep's butter.

biochemical tests led to identifying these isolates as follows: seven isolates of *Lc. lactis* ssp *diacetylactis,* three isolates of *Lc. lactis* ssp *lactis* and three isolates of *Lc. lactis* ssp *cremoris.*

Eleven isolates of cocci picked from M17 agar grew at 15 °C but not at 45 °C. They are heterofermentative; eight of them have the ability to use citrate and only three isolates produced exopolysaccharides (Table 3). All these characteristics, together with the profiles of carbohydrate fermentation, identified three isolates as *Leuconostoc mesenteroides* ssp *dextranicum,* five isolates as *Leuconostoc lactis* and three isolates as *Leuconostoc mesenteroides* ssp *lactis.*

In our study, LAB was isolated from sheep's dhan, a traditional butter made from sheep's milk. The microbiological study of this local product is not documented. Seven LAB species were found which were represented by *Lb. plantarum*, *Lc. lactis* ssp *cremoris*, *Lc. lactis* ssp *lactis, Lc. lactis* ssp *diacetylactis, Ln. lactis, Ln. mesenteroides* ssp *lactis* and *Ln. mesenteroides* ssp *dextranicum.* These results are not in accordance with those reported by Sagdic *et al*. (2002) who isolated LAB from traditional Turkish yayik butter and isolates are represented essentially by strains of *Leuconostoc pseudomesenteroides*, *Leuconostoc gelidum*, *Lactobacillus delbrueckii* ssp*. bulgaricus* and *Enterococcus faecium.* In a previous study, Kacem and Karam (2006) have isolated LAB from smen, it is represented by *Lc. lactis* ssp *cremoris, Lc.lactis* ssp biovar *diacetylactis, E.faecium, Lb.plantarum, Lb.delbrueckii* ssp *bulgaricus, Lactobacillus paracasei* ssp *paracasei, Leuconostoc paramesenteroïdes and Ln.gelidum.*

Table 2
Physiological and biochemical characteristics of *Lactococcus* isolates

Isolates	SB 3	SB 7	SB 12	SB 6	SB 4	SB 11	SB 9	SB 10	SB 8	SB 5	SB 13	SB 14	SB 15
Gram Strain	+	+	+	+	+	+	+	+	+	+	+	+	+
Cell Shape	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Catalase test	−	−	−	−	−	−	−	−	−	−	−	−	−
Growth:													
10 °C	+	+	+	+	+	+	+	+	+	+	+	+	+
40 °C	+	+	+	+	+	+	+	+	+	+	+	+	+
45 °C	−	−	−	−	−	−	−	−	−	−	−	−	−
4% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+
6.5% NaCl	−	−	−	−	+	−	+	−	−	−	+	−	−
Sherman test													
0.1% methylene blue	+	+	+	+	+	+	+	+	+	+	+	+	+
0.3% methylene blue	+	+	+	+	+	+	+	+	+	+	+	+	+
Arginine hydrolysis	−	−	−	+	+	+	+	+	+	+	+	+	+
Fermentative type	h	h	h	h	h	h	h	h	h	h	h	h	h
Acetoin	−	−	−	−	−	−	+	+	+	+	+	+	+
Haemolysis type	γ	γ	γ	γ	γ	γ	γ	γ	γ	γ	γ	γ	Γ
Reductase	+	+	+	+	+	+	+	+	+	+	+	+	+
Fermentation of													
Arabinose	−	−	−	−	−	−	−	−	−	−	−	−	−
Rhamnose	−	−	−	−	−	−	−	−	−	−	−	−	−
Galactose	+/−	−	−	−	−	−	−	−	−	−	−	−	−
Inulin	−	−	−	−	−	−	−	−	−	−	−	−	−
Mannitol	+	+	+	+	+	+	+	+	+	+/−	−	+	+
Sorbitol	−	−	−	−	−	−	−	−	−	−	−	−	−
Lactose	+	+	+	+	+	+/−	+	+	+	+	+	+	+
Saccharose	+/−	−	−	+/−	−	+/−	+/−	−	−	+/−	−	−	+/−
Maltose	−	−	−	−	−	−	−	−	+/−	−	+/−	−	−
Raffinose	−	−	−	−	−	−	−	−	−	−	−	−	−
Starch	+	+	+/−	+	+	+	+	+	+	+	+	+	+
Glycerol	−	−	−	−	−	−	−	−	−	−	−	−	−
Identified as	*Lactococcus lactis* ssp *cremoris*			*Lactococcus lactis* ssp *lactis*			*Lactococcus lactis* ssp *diacetylactis*						

h: homofermentative; n: not determine; SB: sheep's butter.

Table 3
Physiological and biochemical characteristics of *Leuconostoc* isolates

Isolates	SB 22	SB 17	SB 26	SB 19	SB 24	SB 21	SB 1	SB 23	SB 20	SB 25	SB 18
Gram Strain	+	+	+	+	+	+	+	+	+	+	+
Cell Shape	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Catalase test	−	−	−	−	−	−	−	−	−	−	−
Growth:											
10 °C	+	+	+	+	+	+	+	+	+	+	+
40 °C	+	+	+	+	+	−	−	−	+	+	+
45 °C	−	−	−	−	−	−	−	−	−	−	−
4% NaCl	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
6.5% NaCl	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Arginine hydrolysis	−	−	−	−	−	−	+	−	+	−	−
Fermentative type	het	het	het	het	het	het	het	het	het	het	het
Citratase	+	+	+	+	+	+	+	+	−	−	−
Exopolysaccharides production	−	−	−	−	−	−	−	−	+	+	+
Fermentation of											
Arabinose	−	−	−	−	−	−	−	−	−	−	−
Rhamnose	+	−	+/−	−	+	−	−	−	−	−	−
Galactose	−	−	−	−	−	−	−	−	−	−	−
Inulin	−	−	−	−	−	−	−	−	−	−	−
Mannitol	+	+	+	+	+	+	+	+	+	+/−	−
Sorbitol	−	−	−	−	−	−	−	−	−	−	−
Lactose	+	+	+	+	+	+/−	+	+	+	+	+
Saccharose	+/−	−	−	−	−	+/−	+/−	−	+	+	+
Maltose	+/−	−	+	−	+/−	−	−	−	+/−	−	+/−
Raffinose	−	−	−	−	−	−	−	−	+	+/−	−
Starch	+	+	+/−	+	+	+	+	+	+	+	+
Glycerol	−	−	−	−	−	−	−	−	−	−	−
Identified as	*Leuconostoc lactis*					*Leuconostoc mesenteroides* ssp *lactis*			*Leuconostoc mesenteroides* ssp *dextranicum*		

het: heterofermentative; nd: not determined; SB: sheep's butter.

From the results presented here, it is clear that lactococci are the dominant LAB in sheep's dhan. These results are in accordance with various reports indicating that the predominant lactic acid bacteria in butter was represented by *Lc. lactis* ssp. *cremoris* and *Lc. lactis* ssp. *diacetylactis* (Guiraud, 1998; Beerens and Luquet, 1987).

In a previous study, Idoui and Karam (2008) reported that *Lb.plantarum* is the dominant isolate in Jijel's traditional butter, a traditional butter made from cows' milk. In the same way, Benkerroum and Tamime (2004) have reported that *Lb.plantarum* is the dominant LAB in Maroccan smen.

Lactococcus and *Leuconostoc* were the two genera found in the three dhan samples. They represented 50 % and 42.31% respectively of the total isolates, while *Lc. lactis* ssp. *diacetylactis* is the dominant strain (26.92%). This strain is very beneficial for traditional and industrial butter; it produces diacethyl which is the major flavoring component in the final product (Schieberle *et al*., 1993).

3.2. Acid production

The time it took for the milk to coagulate was used to classify the isolated strains as fast, intermediate and slow acidifying strains. The milk inoculated with pure LAB strains coagulated after 6 to 12 hours (table 4). Our results showed that *Lb. plantarum* SB16 and SB2 acidify skim milk faster than any other strain of LAB tested for this property. A slight similarity was observed between the acidification profiles of different *Lactococcus lactis* ssp *lactis* strains and the other strains of *Leuconostoc.* The slowest acidification agent was the isolate *Lc. lactis* ssp *cremoris* SB3; it produced 85°D after incubation for 24 hours. The results of the study conducted by Idoui and Karam (2008) indicated that *Lb.plantarum* and *Lb.curvatus* isolated from Jijel's traditional butter made from cows' milk are the fastest acid producers.

In the study by Haddadin (2005), *Lb.plantarum* was the fastest acid producing isolated strain. The same author has reported that one isolate of *Lc.lactis* was the fastest to coagulate milk with coagulation obtained after six hours of incubation at 30 °C.

3.3. Exopolysoccharides production

The type and character of starter organisms that are used in the production of fermented milks are two of the most important factors in determining the overall quality of the final product. The essential

Table 4
Production of lactic acid by some lactic acid bacteria isolates

	Acidity (°D)				
Time of incubation (hours)	0	6	12	18	24
Lactococcus lactis ssp *lactis* SB 6	18.00 ± 0.00	38.00 ± 0.07	58.20 ± 0.50	62.00 ± 1.00	90.00 ± 0.69
Lactococcus lactis ssp *lactis* SB4	18.00 ± 0.00	39.00 ± 0.09	59.50 ± 0.08	65.80 ± 0.8	93.50 ± 0.90
Lactococcus lactis ssp *lactis* SB 11	18.00 ± 0.00	43.00 ± 1.00	63.00 ± 0.70	70.50 ± 1.60	96.20 ± 0.80
Leuconostoc lactis SB 22	18.00 ± 0.00	42.10 ± 1.10	65.00 ± 0.20	70.10 ± 1.50	98.50 ± 0.00
Leuconostoc lactis SB26	18.00 ± 0.00	39.00 ± 1.00	56.00 ± 0.00	63.50 ± 1.20	90.00 ± 1.20
Leuconostoc lactis SB24	18.00 ± 0.00	38.00 ± 0.90	56.50 ± 0.90	69.50 ± 1.30	92.00 ± 1.23
Lactococcus lactis ssp *cremoris* SB3	18.00 ± 0.00	39.10 ± 1.10	51.50 ± 0.80	61.00 ± 0.80	85.00 ± 1.56
Lactobacillus plantarum SB16	18.00 ± 0.00	52.00 ± 1.30	69.00 ± 2.10	83.50 ± 2.10	110.20 ± 2.50
Lactobacillus plantarum SB2	18.00 ± 0.00	56.20 ± 1.20	70.00 ± 2.30	88.00 ± 2.40	120.00 ± 3.10

criteria for starter selection include acidification, flavor and texture. Three strains, *Ln. mesenteroides ssp dextranicum* SB18, SB20 and SB25, showed their ability to produce exopolysaccharides on M17 media containing 20g.L^{-1}sucrose. The colonies were clearly distinguished (large and pink) according to their different morphological appearance upon growth in hypersaccharose agar. These isolates have interesting textural technological aptitudes. These results were also described by Colman and Ball (1984) and Idoui and Karam (2008). In a previous study, Bouzar *et al*. (1996) reported that *Lb.delbrueckii* ssp.*bulgaricus* CNRZ 1187 and two variants from it produced different yields of neutral heteropolysaccharides when grown in milk.

The production of exopolysaccharides has been documented by Zambou Ngoufack *et al*. (2004), who reported this feature in several gram-positive and gram-negative bacteria, including LABproduction. Broadbent *et al*. (2003) indicated that the incorporation of exopolysaccharide producing cultures into dairy foods can provide viscosifying, stabilizing and water binding functions. It was also reported that the strains producing EPS play beneficial roles in the rheological behavior and texture of the fermented milks (Sutherland, 1990).

3.4. Proteolytic activity

As shown in table 5, the values of the diameter of the proteolysis zone of LAB strains ranged from 5.5 and 7.0 mm after incubation for 24 hours. The isolates were able to grow on YMA media where bacterial proteolytic activity led to clear zones. *Lc. lactis* ssp *lactis* SB11 showed the highest activity (7mm) while *Ln. mesenteroides* ssp *dextranicum* SB20 strain showed the lowest activity (5mm). Twelve out of 26 isolates were able to grow in the same media (YMA) but they showed no proteolytic activity. These results are in agreement with those found by Idoui and Karam (2008) who reported that LAB isolated from Jijel's traditional butter made from cows' milk has proteolytic activity. Peterson *et al*. (1990) reported that important differences exist between species of LAB in terms of the types and quantities of peptidase activities. The results of the study conducted by Haddadin (2005) indicated that all isolated strains had an important nutritional request for easily assimilated nitrogen compounds. The weak proteolytic activities of the genus *Lactobacillus* do not permit protein hydrolysis, casein in particular, in order to obtain low molecular weight compounds.

Table 5
Proteolytic activity of some lactic acid bacteria

Isolates	Diameter of proteolysis zone (mm)
Lactococcus lactis ssp *lactis* SB 6	6.6
Lactococcus lactis ssp *lactis* SB4	6.0
Lactococcus lactis ssp *lactis* SB 11	7.0
Leuconostoc lactis SB 22	5.5
Leuconostoc lactis SB26	6.1
Leuconostoc lactis SB24	6.2
Lactococcus lactis ssp *cremoris* SB3	6.5
Lactobacillus plantarum SB16	6.0
Lactobacillus plantarum SB2	5.5
Leuconostoc mesenteroides ssp *dextranicum* SB20	5.0
Lactococcus lactis ssp *diacetylactis* SB8	6.0
Lactococcus lactis ssp *diacetylactis* SB15	6.2
Leuconostoc mesenteroides ssp *lactis* SB21	6.0
Leuconostoc mesenteroides ssp *lactis* SB1	5.9

4. CONCLUSION

Results obtained in this study showed that sheep's butter contained a diversity of LAB and twenty-six isolates were identified; *Lactococcus* and *Leuconostoc* were the predominant genus in this product. The microbiological study of this local product is not documented, so the results give an idea about the lactic flora in this traditional product.

The results of the assessment of the technological aptitude indicated that the acidification kinetic confirm the weak production of lactic acid by some isolates; some of them showed a good proteolysis activity and only *Ln. mesenteroides* ssp. *dextranicum* isolates were able to produce exopolysaccharide. For future applications, we would select the best combinations amongstrains which would permit the manufacturing of traditional or industrial butter.

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