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Identification of Lactic Acid Bacteria from Algerian Traditional Cheese, El-Klila

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Abstract: The lactic acid bacteria from dried El-Klila, an Algerian traditional cheese were studied, The cheese was also examined for chemical and physical characteristics. The isolated strains from sample K1 belonged to *Enterococcus faecalis*, *Enterococcus faecium*, *Lactobacillus confusus* and *Streptococcus* sp. Enterococci were the most frequently found. However, the isolates from sample K3 were identified as *Pediococcus* sp, *Pediococcus acidilactici*, *Lactobacillus* sp, *Streptococcus* sp and *Leuconostoc* sp. Pediococci were the predominant strains. The samples had high protein content (538·5 g kg^{-1} for K1 and 549·8 g kg^{-1} for K3) and considered as extra-hard cheese.

Key words: Algerian traditional cheese, El-Klila, lactic acid bacteria.

INTRODUCTION

Cheese and fermented milks are among nature's most important contributions to civilisation (Kosikowski 1982). They are made from low cost materials, and provide healthful nutritional elements. The evolution of fermented milk products began many centuries before Christ, probably in the warm climate of the Mediterranean Sea Basin (Kosikowski 1982). The art of cheese making has been developed with recipes handed down from mother to daughter, using the milk of domesticated animals (Macrae *et al* 1993).

In Algeria, El-Klila, a traditional cheese which is popular in the country side, is made from the raw unpasteurised cow or goat surplus milk. The cheese is manufactured by keeping the milk in clean non-sterile pots at room temperature (generally for 2 days) until it tastes sour. The sour milk, called 'Raib', is shaken in special goat-skin receptacles for 2–3 h, then water is added to separate the butter which is collected. After heating the low fat milk for about 15 min at 40–50°C, the whey is separated from the curd by filtration through a gauze sheet. The cheese is consumed in this form or sun dried for long storage.

El-Klila cheese fermentation, like many traditional fermenting processes, is spontaneous and uncontrolled and so involves several food microorganisms whose types are influenced by the environmental conditions of the area where the cheese is produced. Microorganisms which are responsible for the acid production in cheese making are lactic acid bacteria (LAB). They are of great industrial and commercial importance as starter cultures (Cogan 1980). The major end-product of their metabolism, lactic acid, functions directly as antagonist for unwanted bacteria (Kashet 1987; Batish *et al* 1990). In addition the LAB are known to produce proteinaceous inhibitors or bacteriocins (Klaenhammer 1988; Mathieu *et al* 1993). Also, several investigations recently have reported on the antimutagenicity and antitumour activity of several species of these bacteria (Hosono *et al* 1989; Zhang and Ohta 1990). It is likely that only certain strains of LAB have this effect. These strains should be identified and characterised for their effective uses. The widespread nature of these organisms suggest that many sources could be examined in a search for effective strains; however, there are many traditional foods which have been left unexplored.

The purpose of this study was to identify for the first time the lactic acid bacteria involved in the natural fermentation of two different samples of dried El-Klila cheese.

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MATERIALS AND METHODS

Cheese samples

Two dried samples were collected in Algeria by Dr Haddi Mohammed L (Constantine Univ, Algeria). The samples were from different towns; K1 was produced in Setif and K3 in Batna. The cheese was about 4 weeks old from the time of production, and had been kept in a cold room at 4°C until used.

Isolation of lactic acid bacteria

Two of the samples (10 g) of El-Klila cheese were ground in a sterilised mortar and cultured in 100 ml litre^{-1} sterile skim milk at 30°C for 48 h. After serially diluting in 1 g litre^{-1} polypeptone water (Wako, Osaka, Japan), aliquots (100 μl) of the dilutions were spread on sterile petri dishes of MRS agar (Merck Ind, Darmstadt, Germany) supplemented with 1 g litre^{-1} sorbic acid (MRSS, pH 5·7) to suppress the growth of other organisms (Reuter *et al* 1983). Incubation was carried out anaerobically using the Gas Pak system (BBL, Cockeysville, MD, USA) at 30°C for 48 h. The total counts were made and 30 colonies were selected randomly from each sample, subcultured in MRS broth at pH 6·8 and purified by streaking on Bromo Cresol Purple Plate Count Agar (Nisui, Tokyo, Japan).

Bacterial identification

The identification work was done according to the methods described in *Bergey's Manual* (Garvie 1986), and *The Prokaryotes* (Holzapfel and Schillinger 1992). All the strains were maintained by weekly subculturing on MRS agar. All tests were initiated by inoculating from 48 h MRS agar cultures. The cultures were examined microscopically by staining and morphological characteristics noted. Cells were Gram-stained by the method of Harigon and MacCane (1976) with decolorization by ethanol/acetone (50 : 1, v/v).

Growth characteristics were monitored daily at 15, 30, 45 and 50°C in tubes of MRS broth over a 7 day period. Salt tolerance was assessed after 3 days of incubation at concentrations of 40 and 65 g litre^{-1} NaCl in MRS broth.

To detect catalase activity, a drop of MRS broth culture was transferred onto a clean slide, flooded with a drop of H_2O_2 and observed for the production of effervescence.

Gas production from glucose was assessed in MRS broth, using inverted Durham tubes. The inoculated tubes were examined for the production of gas after three days of incubation at 37°C.

Production of ammonia from arginine was done according to the method described by AbdEl-Malek and Gibson (1948).

Nitrate reduction was done as described by Gerhardt *et al* (1981).

Initial pH for growth was tested using sterilised citrate malate broth (CMB) adjusted aseptically with 1 M NaOH. The results were noted after 3 days of incubation at 37°C.

Dextran formation from glucose was tested in medium containing (g litre^{-1}) sucrose 20, yeast extract 10, peptone 10, sodium acetate 5, Tween 80 0·5, $MgSO_4 . 7H_2O$ 0·2, Mn 0·01, Fe 0·01, NaCl 0·01 and 12 agar in distilled water. The plates were observed for the formation of mucoid bacterial growth over 7 days.

Alcohol tolerance was tested by adding ethanol to sterilised MRS medium at final concentration of 100 ml litre^{-1} and observing the growth characteristics for 3 days.

Litmus milk reaction was conducted using litmus medium (Difco, MI, US). Acid production from carbohydrates was tested in MRS broth, but glucose and meat extract were omitted, and 0·04 g litre^{-1} chlorophenol red was added. Solutions (100 g litre^{-1}) of the test carbohydrates were sterilised by membrane filtration (ϕ: 13 mm; pore size: 0·45 μm, Adventec, Tokyo), and added to sterilised medium at a final concentration of 20 g litre^{-1}. The determination of the optical types of lactic acid was performed as described by Okada *et al* (1978), using the following chemicals: D-lactic acid dehydrogenase (from *Lactobacillus leichmanii*), L-lactic acid dehydrogenase (from rabbit muscle) and β-NAD (Sigma, St Louis, MO, USA).

Chemical and physical characteristics of El-Klila cheese

The total nitrogen and total lipid of the cheese samples were determined using standard analytical methods (AOAC 1984). pH of the blended cheese samples was measured using a Horiba D-13 pH meter (Japan) according to the method of Kosikowski (1982). Titratable acidity (as g kg^{-1} of lactic acid) was measured using method of Nout *et al* (1989). Sodium chloride content was determined using a volumetric method (AOAC 1984). Moisture in cheese was determined by drying 1 g duplicate samples in an atmospheric oven at 105°C for 24 h. All the data were analysed with commercial statistical analysis software, PCSAS (SAS, Epson PC).

RESULTS

All 60 isolated strains, with two exceptions, were Gram-positive and, with four exceptions, catalase negative. The Gram-negative and catalase-positive strains were regarded as non-LAB (Sharpe 1979) and were not tested further.

TABLE 1
Physiological and biochemical characteristics of lactic acid bacteria isolated from El-Klila cheese

Characteristics	*Identified groups*[a]									
	K1-I	*K1-II*	*K1-III*	*K1-IV*	*K3-I*	*K3-II*	*K3-III*	*K3-IV*	*K3-V*	*K3-VI*
Hydrolysis of arginine	+	+	+	+	+	+	+	+	−	+
Gas production	−	−	+	−	−	−	−	−	+	−
Nitrate reduction	−	−	−	−	−	−	−	−	−	−
Growth at										
15, 30, 37 and 45°C	+	+	+	+	+	+	+	+	+	+
50°C	−	−	−	−	−	−	−	−	−	+
Growth at pH 4·2 in CMB	NT[b]	NT	NT	NT	+	+	d[c]	+	+	+
Growth at pH 4·8 in CMB	+	+	+	+	+	+	+	+	+	+
Growth at pH 9·6 in CMB	+	+	+	−	−	+	−	−	−	−
Growth in 40 g litre^{-1} NaCl	+	+	+	−	+	+	+	+	+	+
Growth in 65 g litre^{-1} NaCl	+	+	+	−	+	+	+	+	−	+
Growth in 100 ml litre^{-1} ethanol	NT	NT	NT	NT	d	d	+	+	+	+
Dextran formation	NT	NT	NT	NT	NT	NT	NT	NT	NT	−
Lithmus milk										
Acid clot	+	+	+	+	+	+	+	+	+	+
Reduction	+	+	+	+	+	+	+	+	+	+
Gas	−	−	−	−	−	−	−	−	−	−
Carbohydrate fermentation										
Glucose	+	+	+	+	+	+	+	+	+	+
Ribose	+	+	+	+	+	+	+	−	+	+
Lactose	+	+	−	+	+	+	+	−	+	+
Mannose	+	+	−	+	+	−	+	−	−	−
Sucrose	−	−	+	−	+	−	+	−	−	−
Mannitol	−	−	−	−	+	−	+	−	−	−
Sorbitol	−	−	−	−	d	−	d	−	−	−
Dextrin	+	+	+	+	−	−	+	−	+	d
Maltose	+	+	+	+	+	+	+	d	+	+
Cellobiose	+	+	+	−	+	−	+	+	+	+
Starch	−	−	−	−	+	−	+	−	−	+
Xylose	−	−	+	−	+	−	+	−	−	+
Inositol	−	−	−	−	−	−	−	−	−	−
Galactose	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	−	−	−
Melibiose	d	+	d	−	−	−	−	−	−	−
Arabinose	−	+	−	−	+	+	+	+	+	+
Lactate formed	DL + L	DL + L	DL + L	DL + L	DL + L	DL + L	DL + D	DL	DL + D	DL

[a] See Table 2 for the group identifications.
[b] NT, not tested.
[c] d, detected (weak reaction).

Generally, the LAB isolated on MRSS (Tables 1 and 2) belonged to the genera *Enterococcus*, *Pediococcus*, *Streptococcus*, *Lactobacillus* and *Leuconostoc*.

Most strains (89·9%) isolated from sample K1 were identified as members of the genus *Enterococcus*. Cells were ovoid in shape and arranged in chains, could grow at 65 g litre^{-1} NaCl and at pH 9·6. The putative enterococcal isolates were further classified into two groups, K1-I and K1-III; K1-I isolates could not ferment arabinose and were considered as *Enterococcus faecalis* (92·6%) and K1-II (7·4%) isolates fermented melibiose and arabinose and were identified as *E faecium*. The gas-producing short rods appeared in short chains, and could produce acid from sucrose and xylose. They were considered *Lactobacillus confusus* (3·3%). One isolate (group K1-IV) was characterised as *Streptococcus* sp BK1 (3·3%). It was a coccus in chains, could not grow at pH 9·6 or at high salt concentrations (40–65 g litre^{-1} NaCl). It produced DL + L-lactic acid.

Most of the strains isolated from sample K3 (group K3-IV and group K3-VI) were identified as members of the genus *Pediococcus* (33·3%). The cells formed tetrads and produced DL-lactic acid. Among the LAB only cell division of pediococci occurs alternately in two planes at right angles to form tetrads (Gunther 1959). In comparison with all the species of *Pediococcus* mentioned in

TABLE 2
The group identities and number of isolates

Sample	*Group*	*Identities of isolates*	*Number of isolates*
K1	K1-I	*Enterococcus faecalis*	25
	K1-II	*Enterococcus faecium*	2
	K1-III	*Lactobacillus confusus*	1
	K1-IV	*Streptococcus* sp BK1	1
Total			29
K3	K3-I	*Streptococcus* sp BK3	3
	K3-II	*Enterococcus faecium* BK3	6
	K3-III	*Lactobacillus* sp	4
	K3-IV	*Pediococcus* sp	9
	K3-V	*Leuconostoc* sp	2
	KE-VI	*Pediococcus acidilactici*	1
Total			25

Bergey's Manual (Garvie 1986), the strains which were identified as *Pediococcus* sp (90%) were quite different in the following physiological and biochemical tests. They could grow in the range of 40–65 g litre^{-1} NaCl and in the pH range of 4·2–8·5. Among the carbohydrates tested, only glucose, galactose, cellobiose and arabinose were fermented. One strain (group K3-VI) tolerated high salt concentrations (40–65 g litre^{-1} NaCl), grew at 50°C and in the pH range of 4·2–8·5, and produced acid from ribose, xylose and arabinose; it was considered to be *Pediococcus acidilactici*. The three isolates of the group K3-I were cocci. They grew in 65 g litre^{-1} NaCl, but were unable to grow at pH 9·5 or produce gas from glucose. They could ferment all the carbohydrates tested except dextrin, inositol and melibiose. These were considered to be *Streptococcus* sp BK3. All cocci, ovoid in chains (group K3-II), grew at pH 9·6, 65 g litre^{-1} NaCl, and fermented arabinose there also were considered to be *E faecium* and they represented 20% of total strains isolated. Isolates which were rod shaped (group K3-III), which produced no gas from glucose, grew at 15°C and produced DL + L-lactic acid were identified as homofermentative *Lactobacillus* sp (13·3%). All gas-producing cocci (group K3-V) unable to hydrolyse arginine, or produce dextran from sucrose but produce DL + D-lactic acid were tentatively identified as *Leuconostoc* sp (6·6%).

TABLE 3
Mean values (g kg^{-1}) and standard deviation of pH, moisture, total protein, total fat, lactic acid and salt of El-Klila cheese[a]

Characteristic	*Sample K1*	*Sample K3*
pH	4·71 ± 0·01	4·29 ± 0·03
Moisture	125·30 ± 0·17	125·55 ± 0·43
Total protein	538·56 ± 19·31	549·80 ± 0·00
Total fat	138·43 ± 1·10	210·10 ± 1·38
Lactic acid	42·25 ± 9·96	39·00 ± 1·73
Salt (NaCl)	5·07 ± 1·25	5·51 ± 0·50

[a] Values presented are means ± standard deviation for $n = 2$.

The chemical compositions of the two samples K1 and K2, dried El-Klila cheese (Table 3), were found to be quite different, especially in fat content. Sample K1 had about 138 g kg^{-1} fat and can be considered as low fat cheese (Macrae *et al* 1993). However, sample K3 had about 210 g kg^{-1} fat and can be included in the group of half-fat cheese. Due to the high content of protein, and very low moisture, dried El-Klila cheese can be defined as an extra-hard cheese with a high protein content.

DISCUSSION

Results of the microbiological analyses, obtained from the limited characteristics used in this study, showed that some strains could not be identified to species.

The lactic acid produced from the identified groups was either DL + L, DL + D or DL. It is considered that the judgment of the optical types by the enzymatic methods might be more exact than that by specific rotatory power (Okada *et al* 1978).

The majority of isolates from sample K1 were identified as *Enterococcus*. Enterococci occur commonly on plants, in insects and intestinal tracts of human and animals (Martin and Mundt 1972). This fact indicates that the production of El-Klila cheese from unpasteurised milk, and in an open environment could lead to high accounts of enterococci. Hosono *et al* (1989) reported the presence of *E faecalis* subsp *liquifacieus* in Dadih, a traditional fermented milk in Indonesia. Also, enterococci appeared to be an important group in the microflora of white-brined cheese (Litopoulou-Tzanetaki and Tzanetakis 1992). It was suggested that these bacteria stimulated the growth of other LAB by the hydrolysis of caseins into oligopeptides (Trovatelli *et al* 1987).

All the *Pediococcus* strains were isolated from sample K3. Pediococci occur on great variety of plants and fruits, although only in small numbers (Mundt *et al* 1969). They also occur in proteinaceus foods such as fresh and cured meat, fresh and marinated fish (Blood 1975), and in New Zealand cheddar cheese (Dacre 1958). In the first months of grana cheese, pediococci are the most resistant forms, and still present in the cheese at the time of consumption (Macrae *et al* 1993).

All the isolates from commercial fermented cheeses belonged to the genera *Lactobacillus* and *Lactococcus* while those from dried El-Klila cheese belonged to the genera *Enterococcus*, *Pediococcus*, *Lactobacillus*, *Leuconostoc* and *Streptococcus*. This finding supports the theory that a traditionally fermented milk depends on

the microorganisms in the particular environment region in which it is produced. (Tamime and Robinson 1988). Macrae *et al* (1993) reported that the type of cheese made depends on milk composition which varies from animal to animal and from species to species. El-Klila cheese is produced from cow's milk without addition of starter cultures. It therefore can be assumed that the isolates originated as natural contaminants. In addition, the LAB from each sample differed, even among the same species such as *E faecium* BK1 and *E faecium* BK3. These cheese samples were collected from different towns in Algeria.

As found in this study, the isolated microorganisms are considered to have typical biochemical characteristics. From this aspect, we are now investigating antimicrobial activities of these LAB against several strains of Gram-positive and Gram-negative bacteria. The results will be reported in due course.

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