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Identification of a *Lactococcus lactis* Isolated from a Fresh Local Cheese of the Western Algerian Steppe "J'ben of Naama"

A.A. Dahou[1], A.A. Bekada[2], A. Homrani[1]

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ABSTRACT

Background: The purpose of the study is to evaluate the diversity of lactococci, lactic bacteria, recovered from "J'ben", a local cheese made from the milk of the Rembi sheep breed, a product of exploitation in the Algerian steppe regions of Naama.

Methods: The bacterial species were isolated from samples of the recovered cheese exploitation and analyzed using genotypic methods. The isolation of bacterial DNA from purified *Lactococcus* cultures has been established by an amplification of ribosomal DNA 16s using the specific universal primers of prokaryotes.

Result: The 16s DNA sequencing of all isolates, for genotypic identification, confirmed the predominance of *Lactococcus lactis*. This lactic dominance determines the quality and distinctiveness of this cheese in the region. The results obtained from acidification and proteolysis kinetics met the technological requirements and good functionality, from the strains used individually and in mixed culture to the cheese processing.

Key words: Identification, *Lactococcus lactis*, Local cheese, Rembi sheep.

INTRODUCTION

The microbial flora, natural or native, plays an important role in the quality of the raw milk cheese, in particular in its taste. It allows preserving the typicality and some sensory diversity of cheeses (Montel *et al*., 2014). The local cheese is therefore considered a biodiversity heritage and can be characterized and preserved as a product with protected designation origin (Cibik *et al*., 2010 and Sakore *et al*., 2007).

In Algeria, cheese production is always done in farms owned by families. J'ben is the most popular traditional cheese and its manufacturing method is still in use today, with an increase in its consumption in steppe areas, due to its pleasant organoleptic and nutritional properties produced by an indigenous microflora (Bousbia *et al*., 2018).

The objective of the study is to present the issues of control of microbial communities in ecosystems of cheese from raw milk of a herd of local breed that are aimed at maintaining the qualitative character of the cheese J'ben of steppe areas of the wilaya of Naama.

From this characterization the study was oriented to the species *Lactococcus lactis* identified genotypically to its dominance of the analyzed ecosystem and its highly sought technology skills in transforming cheese especially acidifying and proteolytic processing.

MATERIALS AND METHODS

J'ben manufacturing process

The traditional transformation of raw milk into J'ben cheese is as follows: The collected raw milk is filtered and then left to its own devices in milking cans at a room in a temperature between 22 and 26°C for 18 to 24 hours; the Rayeb obtained as it undergoes skimming - churning in a sheep skin (chekoua). The resulting butter called Semnah is removed in a single clod, the remaining whey called Leben is heated slightly until the whey is separated, the water phase (whey) is separated into a muslin for 10 to 15 hours and the coagulum obtained represents J'ben cheese.

The recovered cheese comes from a local herd, the Rembi sheep; product of an extensive and traditional breeding system. The milk collection is manual, preceded by a cleaning of the udders.

Samples of J'ben cheese

Cheese sampling was based on 15 productions made over the 45-day high lactation period and at a rate of a cheese produced after 3 days of lactating a sheep herd consisting of 85 locally bred Rembi ewes. Samples were collected aseptically in containers sterile operation of the farm in Khabaza village of El-Biodh commune in Naama city and kept at temperatures up to 10°C, in order to be transferred for experimental analysis for the laboratory.

Microbiological analyses

Samples of sufficient mass of J'ben cheese are well done aseptically, 1.5 g of the sample is placed in a sterile tube

[1]Laboratory of Science and Technics of Animal Production, University Abdelhamid Ibn Badis Mostaganem, Algeria.
[2]University Center of Tissemssilet, Algeria.

Corresponding Author: A.A. Dahou, Laboratory of Science and Technics of Animal Production, University Abdelhamid Ibn Badis Mostaganem, Algeria. Email: abdelkader.dahou@univ-mosta.dz

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containing 50 ml of sterile physiological water, incubated one hour at 37°C until massive solubilization. The separation of the two phases is then carried out by centrifugation for 10 minutes at 3500 rpm.

Using a sterile pipette, 1 ml of the aqueous phase constituting the mother dilution is taken and introduced into a tube containing 9 ml of sterile physiological water giving dilution 10^{-2}. The others from 10^{-3} to 10^{-6} are obtained in the same way (Idoui *et al*., 2008).

Enumeration of lactic acid bacteria

The media used for the cultivation of the lactic acid bacteria are respectively the following:

Middle of M17: Tryptone 2.5g, Peptone meat pepsique: 2.5g, Papainique soy peptone:5g, Autolytique yeast extract: 2.5g, Meat extract: 5g, Lactose: 5g, Sodium glycerophosphate: 19 g, Magnesium sulfate: 0.25 g, Ascorbic acid: 0.5 g, Bacteriological agar: 15 g and 1000 ml of a distilled water. pH = 6.5, sterilization in an autoclave at 120°C for 20 minutes. This very rich environment, allows rapid development of all species of lactic bacteria.

The sowing of the *lactococcus* Petri dishes is done in the mass and is carried in an incubation oven at 30°C for 48 hours. The results are expressed in cell numbers per ml (Badis *et al*., 2005).

Isolation and purification

After count, apparent characteristic colonies of bacterial study groups are collected in Petri dishes to study their morphology. The isolated bacteria are found, in shells and diplocoques chains, gram positive, catalase negative and not producing release of oxygen when they are tested on a drop of oxygenated water, they are retained as lactic bacteria. After the cultivation of strains isolated from lactococci on stocks of their aforementioned environments, seeding in streaks on their agar are made and put in incubation at 30°C and at 37°C for 48 hours. Among the well-insulated and purified colonies that appear on Petri dishes, a characteristic colony is removed from each box and stored at 4°C on Eppendorf tubes with M17-15% glycerol for identification (Guiraud, 2003 and Tailliez, 2001).

Genotypic characterization

The identification of lactococci by phenotypic methods is not reliable (Badis *et al*., 2005). Tool was developed, at the level of the laboratory based on molecular biology by an amplification of ribosomal DNA 16s by universal primers specific to prokaryotes, a critical analysis of the resulting sequence and a comparison with the positive control of *Lactococcus lactis* ATTC 49032. These results are consistent with those of Gusils *et al*., 2010 and Reats *et al*., 2011.

Technology skills of *Lactococcus lactis*

The selection of starter strains for cheese processing is based on their acidification power and proteolytic power (Montel *et al*., 2014).

Acidifying power

The acidifying function is the most sought after metabolic property of lactic bacteria used in cheese manufacturing. It manifests itself through the production of lactic acid from the fermentation of carbohydrates during bacterial growth (Montel *et al*., 2014). The measure of acidifying activity is to monitor the pH of the cultures of the 10 isolates of lactococci first, according to time. On the other hand, it is to simultaneously measure the total acidity by soda. To carry out this test; skimmed milk at 10% dry extract was prepared in vials of 250 ml, sterilized at 120°C and cooled to the seeding temperature of 37°C. Vials inoculated with lactic culture (at 1%) was carried out with an incubation at 37°C and a control of acidification kinetics at controlled time intervals of 2 hours, 6 hours, 12 hours and 24 hours. Acidification is controlled by titling 10 ml of milk with Dornic (N/9) soda in the presence of a coloured phenolphthalein indicator.

The acidity is determined by the formula:

$$\text{Acidity (°D)} = V_{NaOH} \times 10$$

Proteolytic power

Proteins are the main source of nitrogen for lactic acid bacteria. The Lactococci develop two types of proteases that hydrolyz casein. Type I proteases proteolysis β caseins and type II proteases lyses β, αs1 and κ caseins. To determine the proteolytic activity of lactic acid bacteria, a PCA agar medium culture supplemented with 10% of skimmed milk was poured, solidified and dried on Petri dishes. Then, sterile wattman paper discs was deposited on the surface of the agar and soaked with a 20 µl volume of a young lactococcus culture. After incubation at 37°C for 24 hours, proteolysis was manifested itself in clear areas around the discs (Savijokie *et al*., 2006 and Veuillemard, 1986).

RESULTS AND DISCUSSION

Microbiological analyses

The results of the microbiological analyses are represented as follows:

Enumeration of lactic acid bacteria

Lactic acid bacteria have been counted in all samples of cheese studied from sheep's milk. Their number varies from 3.10^6 cfu/g to 2.10^8 cfu/g. This difference in number of lactic bacteria in the samples is the result of the variability of the dairy microbial ecosystem within the same breed with a predominance of 80% in lactococci. The number determines the quality of sampling in relation to animal race of a same livestock operation and transformation as described by Guiraud (2003) and Gusils *et al*. (2010).

Isolation and purification

The study focused on lactic acid bacteria of applied interest. The dominance of the lactic bacteria for cheese J'ben sheep was as follows: 80% lactococci (10 isolates), 15% leuconostocs (03 isolates) and 5% pediocoques (01 isolate). 10 purified isolates of lactococci were selected for a

genotypic characterization. Physiological and biochemical tests have shown that all isolates were gram positive and catalase negative, which is the characteristic of lactic bacteria.

In the study, the characterization also focused on certain growth factors, such as incubation temperature and optimal pH. Compared to the growth parameters of lactic acid bacteria, defined by Badis *et al*. (2005)and Guiraud (2003), *Lactococcus* have been determined to develop at a temperature of 25 to 37°C with an optimum at 30°C, an aerobiosis incubation and a tolerance to variations in ion concentrations. These bacteria accept concentrations of up to 6.5% salt but not required for growth. These species of *Lactococcus* confirm their belonging to *Lactococcus lactis*, since in addition to their tolerance of hypersalted environments, they prefer a pH of 6.5 in line with the majority of neutrophil lactic bacteria that develop at an included pH 5.5 to 6.5 with an optimum of 6.5. On the other hand, isolated lactococci have a homo-fermentary lactic fermentation profile (Table 1).

Genotypic characterization

The genotypic study carried out by molecular characterization, based on the amplification and sequencing of bacterial DNA, produced the desired results. The visualization of the profiles, obtained after electrophoresis under ultraviolet light, gave a comparative dendrogram with degrees of similarity to a distinctive witness of *Lactococcus lactis*.

The separation of PCR amplifiers by electrophoresis allowed the visualization of the DNA bands characteristic of the 10 strains identified at a molecular weight of 570 bps (Fig 1). 10 isolates were identified by PCR in applying the technique of the ARNr16s. Obtained profiles have been treated with the Gel Compar II software and compared with the profiles of strains of used references.

Table 1: Biochemical and physiological profile of the identified lactic strains: *Lactococcus lactis.*

Character	Isolated strain: *Lactococcus lactis*
Gram	+
Form	Hull
Catalase	-
Growth at 25°C	+
Growth at 30°C	+
Growth at 37°C	+
Growth to 4.5% of Nacl	+
Growth at 6.5% of Nacl	+
Growth at pH 4.5	-
Growth at pH 5.5	+
Growth at pH 6.5	+
Growth at pH 9.6	-
Type of fermentation	Homo-fermentative

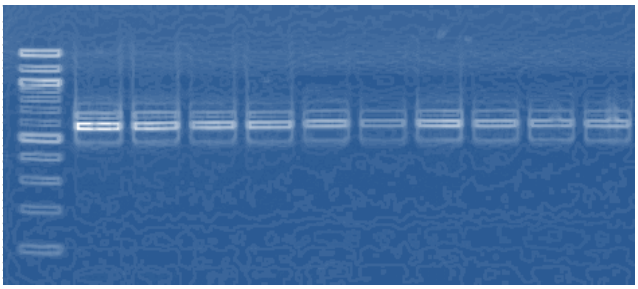

Fig 1: Gel Electrophoresis of the products of the PCR of the 10 isolates of *Lactococcus lactis* viewed by GBOX to 570PB.

Technology skills of *Lactococcus lactis*

Acidifying power

Acidifying activity is one of the main functions of lactic bacteria. From these results, we notice that all of the identified *Lactococcus* present a progressive lactic acid production. The latter is accompanied by a lowering of the pH of the medium.

The species *Lactococcus lactis* : Lc1, Lc4 and Lc8 are considered as being the most acidifying, with a medium amount of lactic acid of 11.8; 11.2 and 10.8 g/l respectively, after 24 hours of incubation. In parallel, the pH values achieved with these strains respectively range between 4.66, 4.72 and 4.75 (Fig 2 and 3).

The results are consistent with those of Cibik *et al*. (2010) and Sakore *et al*. (2007), *Lactococcus lactis* is of

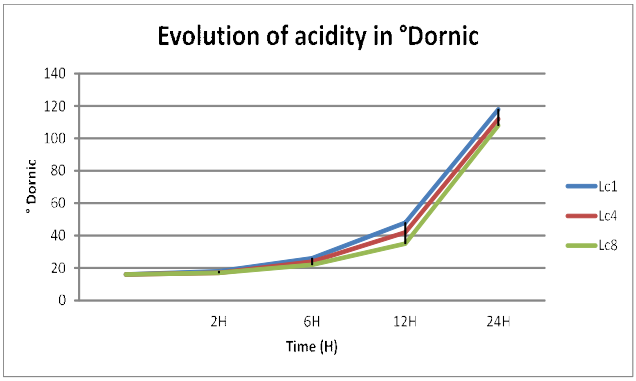

Fig 2: Acidifying power of isolated lactic strains *Lactococcus* Lc1, Lc4 and Lc8: Evolution of the acidity in ° Dornic after 24 H of incubation.

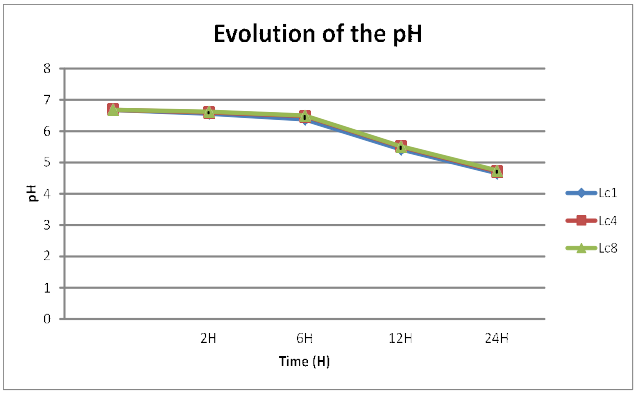

Fig 3: Acidifying power of isolated lactic strains *Lactococcus* Lc1, Lc4 and Lc8: Evolution of the pH after 24 H of incubation.

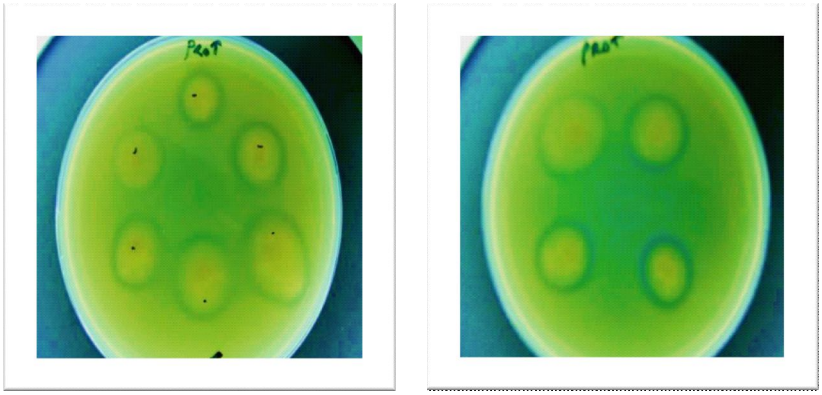

Fig 4: Proteolytic power of 10 *Lactococcus lactis* isolates.

crucial importance for manufacturing dairy products, such as fermented milks and cheeses. When *Lactococcus lactis* is added to milk, the bacterium uses enzymes to produce energy molecules (ATP),from lactose.The byproduct of ATP energy production is lactic acid. The lactic acid produced by the bacterium curdles the milk that then separates to form curds, which are used to produce a fresh cheese with very appreciable nutritional and organoleptic qualities.

Proteolytic power

The results of the test are summarized in the Table 2. Analysis of the results revealed visual proteolytic activity with lysis zones on the agar PCA culture medium mixed with 10% of skimmed milk.

According to Veuillemard, (1986), the strain is called proteolytic if it has an area of lysis of diameter between 5 and 15 mm. In comparison to this data, the lactococci strains in the study were proteolytic with the diameters of the zones of proteolysis were included between 7 and 13 mm (Table 2, Fig 4).

The results are similar to those obtained by Savijokie *et al*. (2006). The proteolytic activity of lactic bacteria is essential for their growth in milk as well as for the development of the organoleptic properties necessary for the maturation of cheeses.

The lactic cheese microflora dominated by *Lactococcus* preserves its typicality induced technological skills generated by acidifying and enzymatic activities that give J'ben Naama appreciable nutritional value by the essential amino acids released by *Lactococcus* species identified lactis to proteolytic character.

The comparison of the results with studies by Cibik *et al*. (2010), Montel *et al*. (2014) and Saha *et al*. (2017), allows to conclude that our country has a rich lactic microbial ecosystem and our traditional dairy products can be a non-negligible source of microbial species, either lactic flora or flora of refining with very interesting technological properties. Rare species such as the *Lactococci* in industrialized countries, are frequent in our traditional cheeses, the fact which contributes to the enrichment of the niche and the knowledge of their ecology.

Table 2: Proteolytic power of identified lactococcus species.

Species identified	Proteolytic power average in mm
LC. lactis Lc1	13
LC. lactis Lc2	6
LC. lactis Lc3	7
LC. lactis Lc4	10
LC. lactis Lc5	8
LC. lactis Lc6	6
LC. lactis Lc7	12
LC. lactis Lc8	9
LC. lactis Lc9	11
LC. lactis Lc10	10

LC : Lactococcus.

CONCLUSION

Our work at the Laboratory and at the farm level on local dairy microbial communities has allowed us to select indigenous ferments, depending on available farming methods, to identify isolated species by omic methods and understand how the microbial ecosystems of J'ben cheese work, in relation to natural flora, cheese practices and the physical-chemical matrix. The purpose of our research is to preserve the public heritage that guarantees local peculiarities on which human activities continue in the often-difficult steppe zones of Algeria where they are based. Our laboratory teams through this type of study contribute to the knowledge of the biodiversity of the microbial ecosystems of our local cheeses, to their mastery and to the preservation of their typicality.

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