

REGULAR ARTICLE

# Potential use of the nisin produced by lactic acid bacteria for longer conservation of Camel cheese

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## ABSTRACT

In fermented foods, lactic acid bacteria (LAB) display numerous antimicrobial activities. This is mainly due to the production of organic acids, but also of other compounds, such as bacteriocins and antifungal peptides. Several bacteriocins with industrial potential have been purified and characterized. This study aims to highlight on the antagonistic effect of lactic strain producer of bacteriocin (*Lactococcus lactis* ssp. *Lactis*) against a psychrotrophic strain (*Pseudomonas fluorescens*) contaminating the milk stored in the cold. The strain producing lactic bacteriocins (nisin-type) is isolated from a fresh camel cheese prepared in the laboratory with a significant yield, equal to 35.5%. The indicator strain is a psychrotrophic strain isolated from a sample of raw camel milk, stored at 4°C for more than three days. Thus, the neutralized supernatant of a culture of *Lactococcus lactis* ssp. *Lactis* in M17 medium (8000 g/20 min at 4°C) was tested against the strain of *Pseudomonas fluorescens* by the three techniques of gel diffusion; spots, wells and disks. The neutralized supernatant showed antibacterial activity which was manifested by the appearance of inhibition zones where diameter differs depending on the test used. Diameters varying between 9 and 18 mm and between 12 and 29 mm were recorded respectively with wells and disks. The technique of spots has been less conclusive.

**Keywords:** Dromedary; *Lactococcus lactis* ssp. *lactis*; Milk; Nisin; *Pseudomonas fluorescens*

## INTRODUCTION

Camel milk, like other mammals, is a medium with a complex chemical and physical composition allowing the camel to meet its energy and nutrient requirements during the first stage of its existence (Kamoun and Ramet, 1989).

Processing camel milk is considered difficult, including cheese making (Faye et al., 1997; Konuspayeva et al., 2014). The ability of a milk cheese processing is closely linked to the nature of its constituents (Goursaud, 1985), Kamoun and Ramet (1989), Akhmetsadykova et al. (2013) and Konuspayeva et al. (2014) have shown the ability to transform this milk into cheese with a satisfactory shelf, if we take into account the peculiarities inherent to its physico-chemical composition. More recently, work undertaken at the University of Ouargla showed that the substitution of enzymes commonly used in cheese (commercial rennet) by gastric proteases from the stomachs of adult camels improves the ability of camel milk to coagulate (Boudjenah-haroun et al., 2011).

On the other hand, during the milk cooling, psychrotrophic bacteria are able to produce thermostable lipolytic and proteolytic enzymes, causing bad taste in cheese (bitter taste, and unwanted off-flavors.) (Le jaouen, 1993). These micro-organisms present in raw milk, have the ability to grow at low temperature (4-8°C). Among the incriminated flora is the genus *Pseudomonas*, especially the species *Pseudomonas fluorescens* (Law and Haandrikman, 1997).

At temperatures below 10°C, lactic milk flora is no longer dominant, and other micro-organisms considered harmful in cheese such as psychrotrophic become dominant (Le Jaouen, 1993). The lactic acid bacteria can produce numerous antimicrobial metabolites such as organic acids, hydrogen, carbon dioxide, reuterin, diacetyl peroxide and bacteriocins. These last are antimicrobial peptides which inhibit the growth of spoilage bacteria or pathogens (Dortu and Thonart, 2009). In food industry, only *Lactococcus lactis*'s nisin is permitted as a food additive (E 234) since 1969 by WHO (World Health Organization). According to the Generally Recognized As Safe (GRAS), nisin inherit

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a broad spectrum of antibacterial activity directed mainly against Gram positive bacteria. Nisin is effective against pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, and *Clostridium tyrobutyricum* (Kalchayanand et al., 2008.).

This study aims to highlight on the antagonistic effect of a lactic bacteriocin producer strain (*Lactococcus lactis ssp. Lactis*) against psychrotrophic strain (*Pseudomonas fluorescens*) which contaminate milk.

## MATERIALS AND METHODS

### Camel milk

The camel milk was collected early morning from a free range camel herd (*Camelus dromedarius*), Sahraoui breed, in good health, living in the South-East Ouargla region (Algeria)

### Preliminary analyzes

The sample of milk was transported in sterile bottles in a cooler to the laboratory for experiencing analysis, physico-chemical (pH, acidity and density) and microbiological (reductase assay).

### Cheese making

Cheese was made with gastric extract enzymes obtained from abomasums of older camel adopting the method described by Valles and Furet (1977). This extract possesses high clotting activity (0.360 RU) and low proteolytic activity (Boudjenah-haroun et al., 2011). Ramet's diagram, 1993 for the production of camel cheese was followed. Curd stay was obtained and the cheese yield was calculated.

### Isolation of nisin-producing strain

Isolation of nisin produced from cheese strain was achieved according Edima (2007). The method involves taking a sample of cheese (10 g) and mixed with 90 ml of citrate buffer at 2%. After crushing with Stomacher for 3 minutes at ambient temperature, the resulting suspension is subjected to successive decimal dilutions in the same buffer until reaching a dilution required for bacterial isolation ( $10^{-8}$ ).

### Identification of the nisin producing strain

Identification of the isolated strain was done through exams: macroscopic (aspects of colonies), microscopic (after staining GRAM), tests (catalase and oxidase) and tests from the gallery of SHERMAN: culture in nutrient broth (45°C, pH 9.6, NaCl 6, 5%) (Guiraud, 1998; Joffin and Leyral, 2001).

### Protocol of nisin production

The production protocol used was modeled on those made by several authors (Barefoot et al, 1983; Lachance, 2000;

Sebti, 2002; Doumandji et al, 2010). Nisin production started with a pre-culture of the strain *Lc.lactis ssp. lactis* previously isolated. First, the pre-culture was obtained by inoculating sterile test tubes containing 9 ml of M17 broth with 1ml of culture of more than 24 hours of the strain *Lc.lactis ssp. Lactis*, isolated from camel milk cheese, in the same broth. The tubes were incubated at 30°C with shaking at 140 rpm for 18 h in an incubator shaker. To facilitate the diffusion of the bacteriocin of *Lactococcus lactis*, M17 medium was used. 100 ml of this medium was divided into two Erlen Meyers (100 ml), enriched with 1% glucose and sterilized for 20 minutes at 120°C. Then the M17 medium was inoculated with 10% of the pre-culture *Lc.lactis ssp. lactis*, and the culture was incubated for 18 to 24 h at 30°C with shaking at 140 rpm. For recovery of the supernatant, the bacterial culture was distributed in hemolysis tubes (5 ml) and centrifuged at 8000 g for 20 min using a refrigerated centrifuge; the supernatant was carefully recovered with removal bacterial cells collected from the bottom of the tubes. To eliminate the effect of organic acids, the pH of the supernatant was adjusted to 6 with NaOH (1N).

### Isolation and identification of the target strain *Pseudomonas fluorescens*

Psychotrophic strain *Pseudomonas fluorescens* was isolated from a milk sample stored at 7°C for 10 days according to Milliere and Veillet-poncet (1979).

The identification of the isolated strain was achieved by macroscopic and microscopic examination (fresh state and after Gram staining), physiological tests such as growth in nutrient broth at different temperatures (4°C, 43°C) and in media: King A and King B. identification was performed using an API 20 E miniaturized gallery (Biomérieux) (Guiraud, 1998; Vezina and Lacroix,2000).

### Study of the inhibitory activity of nisin against *Pseudomonas fluorescens*

The antimicrobial activity of the supernatant recovered from culture of *Lactococcus lactis subsp. lactis* was tested using various methods of gel diffusion technique recommended by Tag et al. (1976). It was based on the appearance of a zone of inhibition caused by the culture supernatant containing the bacteriocin, deposited in wells, or even soaked on spots or disks.

The inoculum of the test strain (*Pseudomonas fluorescens*) was prepared in sterile test tubes on inoculating 5 ml of sterile saline with a colony of the test strain taking from nutrient agar medium. Three Petri dishes containing Mueller Hilton medium (MHA) were inoculated by the target organism (*Pseudomonas fluorescens*). On each box dug wells using the tip of a sterile Pasteur pipette (4.5 mm diameter) were filled

by 50 µl of supernatant. The Petri dishes were prepared and pre-incubated for 2-4 hours at 4°C to allow the radial diffusion of the inhibitor. Incubation is then carried out at 30°C for 18 to 24 hours under aerobic conditions. For spots method, 10 µl of the supernatant to be tested were deposited on the surface of the agar using a micropipette. The dishes were steamed for 18 to 24h at 30°C.

The desired result was manifested by the presence of clear zones of inhibition in cloudy water formed by the growth of the target strain (Izquierdo, 2009). Then, for discs method according to Tadesse (2004).

- Flooding the surface of the Petri dishes containing Mueller-Hilton medium by inoculum of the target strain
- drying the dishes for 30 min at 30° C. After drying, deposited on the surface of the agar discs sterile filter paper previously impregnated by bacteriocin supernatant, then once dry even boxes for 30 min,
- Placing the boxes at 4° C for 4h ensured the dissemination of the bacteriocin, and then incubating the dishes at 30° C for 24 hours.

Reading the bacteriocin's activity was done by measuring the diameter of inhibition around wells or inhibitory zones (Iz) in mm (Allouache et al., 2010). Inhibition was considered positive if the diameter (Iz) was larger than 2 mm according to Doumandji et al. (2010). Measuring the inhibition diameter (Iz) was performed according to the following formula.

$Iz \text{ (mm)} = \text{diameter of the inhibition zone obtained (mm)} - \text{diameter of wells (4,5 mm)}$

The antibacterial activity in arbitrary units per ml (AU/ml), according to Champagne (2007), was calculated from the highest dilution (D) where there was still the presence of an inhibition of more than 2 area mm, according to the following equation:

$$A \text{ (UA/ml)} = (1000/10 \mu\text{l}) (1/D)$$

## RESULTS

### Physico-chemical analyses

Camel milk samples analyzed are apostrophized by a titratable acidity of about 17, 8 ± 0.5 °D. The values of their density and pH at (T=20°C) are respectively 1, 028± 0.001 and 6, 56± 0.002.

### Bacteriological quality of camel milk

Discoloration of methylene blue of the analyzed camel milk sample occurred after a longer than 4 hours. This milk had a good bacteriological quality. It contained less than

2.10<sup>6</sup> cells/ml by referring to the grid of Larpent, 1970 and Guiraud, 1998.

### Isolation and identification of bacterial strains

#### *Isolation and Identification of Lactococcus lactis ssp. lactis*

The isolated strain was devoid of catalase and oxidase. It was immobile. It ferments glucose giving only lactic acid (homofermentative). It cannot hydrolysis neither arginine nor citrate. It grew at pH 9,6 and 45°C. The results of physiological and biochemical tests were used to identify the isolated strain as a strain belonging to the species *Lactococcus lactis ssp. lactis* (Guiraud, 1998).

#### *Isolation and Identification of Pseudomonas fluorescens*

The isolated strain was catalase positive, oxidase positive, and did not react with methyl red (RM negative reaction). It degraded glucose, arabinose and melibiose, aerobically on the gallery API 20 E. Also, it hydrolyzed arginine and gelatin in the same gallery. Growth on nutrient broth (BN) was recorded at 4°C and not at 43°C. Pyoverdine production (pigment) was displayed on King B (fluorescent green) environment. The morphological characters and different biochemical and physiological tests could identify isolated strain as belonging to the species *Pseudomonas fluorescens* strain (Guiraud, 1998).

### Study of the antibacterial activity of the extract of nisin on *Pseudomonas fluorescens*

The measurement of average diameters of inhibition zones (Iz) for Wells and Discs method were respectively 8, 75 ± 0, 01 (for 4 Wells positives) and 20, 05± 0,015 (for 10 Discs positives).

The method of critical dilution of supernatant was used to calculate the antibacterial activity in arbitrary units (AU) according to equation of Champagne (2007).

$$A \text{ (UA/ml)} = (1000/10 \mu\text{l}) \times (1/D).$$

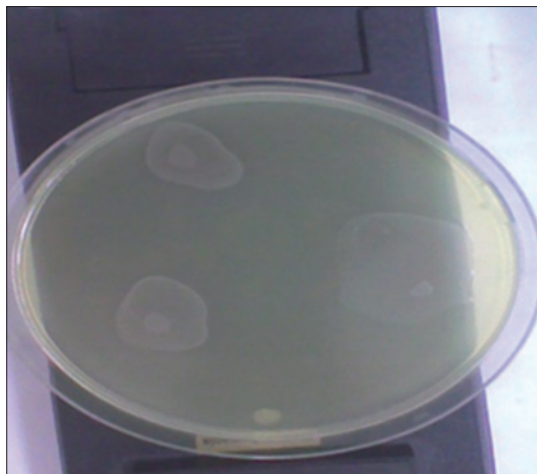
Since maximum dilution to give a further inhibition of a diameter greater than 2 mm by the method of spot area is the 1/32 dilution.

$$A \text{ (UA/ml)} = 1000/10 \times 1/1/32$$

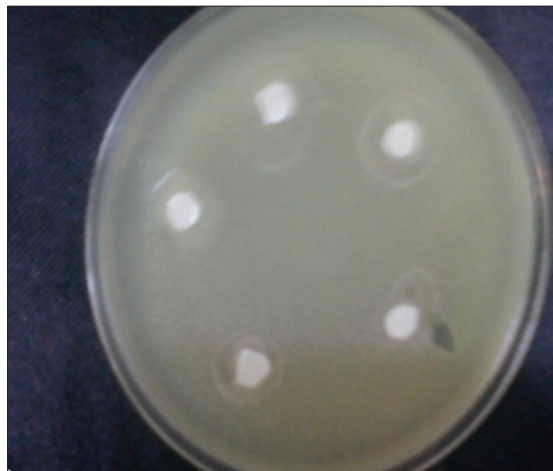
$$A = 3200 \text{ UA/ml}$$

## DISCUSSION

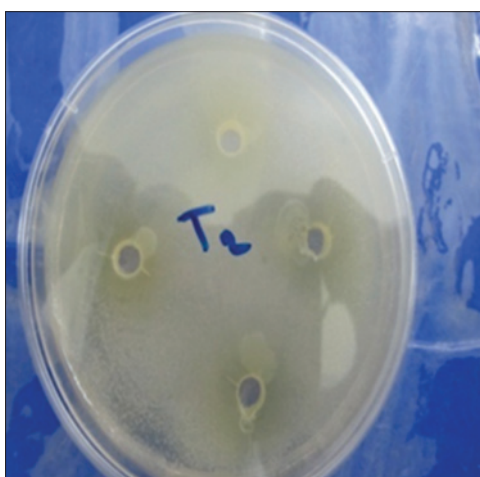
The three methods of gel diffusion gave positive results with the appearance of inhibition zones (Iz) distinctive (apparent halos) around spots, wells and disks. The results were illustrated by Figs. 1, 2 and 3. The (Iz) had varying



**Fig 1.** Antibacterial activity of supernatant against *Ps fluorescens* by the method of spots.



**Fig 2.** Antibacterial activity of supernatant against *Ps fluorescens* by the disc method.



**Fig 3.** Antibacterial activity of supernatant against *Ps fluorescens* by the method of well.

diameters. This study showed also that the method of spots was less suitable. This could be due to the poverty of supernatant's distribution or to the insufficient diffusion

of bacteriocin. The estimated using the critical dilution of antibacterial activity used by Mayr (1972) was equal to 3200 AU/ml. This may be more or less accepted, Meghroun et al. (1999) reported that the estimation of the activity of a bacteriocin by the method of diffusion in agar was a relative measure, because the sensitivity of the target bacteria, the growth medium used, the concentration of the test agar medium, and the concentration of bacteriocin were all factors that may affect results.

Generally, bacteriocins of lactic acid bacteria were not active against Gram-negative bacteria. This was due to the difference in the composition of the cell envelope of Gram positive and Gram negative bacteria. However, some studies suggested that changes in permeability properties of the outer membrane after some treatments used (the addition of EDTA, lysozyme.) in combination with bacteriocins or stress conditions would make sensitive Gram-negative bacteria to bacteriocins (Abee, 1995; Cintas et al., 2001; Deegan et al., 2006). At our knowledge, no studies on the effect of nisin used only on *Pseudomonas* have been reported in the literature. However, the spectrum of activity of some bacteriocins (such as nisin) according to Klaenhammer (1993) may not be restricted to taxonomically related species or even occupying the same ecological place of the producing bacteria. From a practical point of view, the field of activity of a bacteriocin may be larger or smaller depending on the environmental conditions and the concentration of active substance.

## CONCLUSION

This study was contributing to improve the keeping quality of camel milk usually consumed fresh and also to master the technique of its transformation into a cheese complies with the requirements of the consumer, by reducing its psychrotrophic flora responsible for bitterness defects and bad tastes. It is possible to use nisin produced by a strain of camel origin for the preservation of chilled camel milk. Finally, the isolation of lactic acid bacteria especially from cheese or camel milk and highlighting their antagonistic power production of bacteriocins against Gram-negative flora, constituted an interesting topic to extend because little informations are available in the literature on these microorganisms.

## Authors' contributions

W. Souid is the principal author of this work. She has made major contributions to this article in sampling, physicochemical analysis of milk samples, identification isolation etc.). S. Boudjenah-Haroun was Involved in the production of the cheese using the enzyme extracts coagulants of dromedary also in conception and design

of study. OK. Siboukeur Was Involved in microbiological analysis of milk samples and bacterial isolation and identification. A. Mati Was Involved in supervisor of the research project which is affiliated to his laboratory.

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