

Antagonistic Effect of the Lactic Bacteria Isolated from the Camel Milk on *Staphylococcus aureus* in Yoghourt Manufacturing

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Abstract: This paper deals with the lactic bacteria found in the raw camel milk producing antibacterial substances. Samples of milk were obtained from female camels of herds from nomads living in the south of Algeria. The antibacterial activity of the bacteria was tested on *Staphylococcus aureus* strains and to highlight this activity in a yoghurt for therapeutic purpose.

Among the seven (07) strains of lactic bacteria which were isolated from camel milk *Lb. fermentum* (Lc17) presented the highest antagonistic effect on *S. aureus*. After incorporation in the yoghurt, it was verified that there was no interaction between the lactic ferments of yoghurt and *Lb. fermentum* and no modification of the acidity. However the realization of a yoghurt containing *Lb. fermentum* with shown a total inhibition of *Staphylococcus aureus* to a load 5,87 log UFC compared to that of yoghurt made up exclusively of lactic leavens, after 4 hours of incubation in mixed culture.

Keywords: Antagonism, lactic bacteria, Therapeutic purpose, camel, *Staphylococcus aureus*, yoghurt.

INTRODUCTION

The lactic acid bacteria has received considerable interest because their antagonistic properties make them particularly useful as biological preservatives [1]. The microflora of bovine milk has been extensively studied, however that of camel milk, has received much less attention in the peer-reviewed literature [2].

Many strains of lactic bacteria are currently being studied to evaluate their utility in the prevention or cure of diseases associated with the consumption of yoghurt. Beneficial effects have been detected regarding: digestion of lactose [3], symptoms of diarrhea [4], inflammatory bowel diseases [5] and, possibly, gastric ulcers [6] and the regulation of the blood lipid level [7].

OBJECTIVES

The aim of this work was isolating new strains of bacteria from camel milk able to inhibiting pathogenic bacteria such as *Staphylococcus aureus* and demonstrated this activity in dairy 'yogurt' for therapeutic purposes

Isolation of Lactic Acid Bacteria from Camel Milk

Milk Collection

Samples of raw camel milk coming from the Djelfa region (south of Algeria) were collected. Each one was

constituted by 150 ml of milk collected directly from the breast of the animal.

Decimal dilutions were prepared using sterile Ringer solution. A volume of 0.1 ml of each dilution was used for double layer surface plating on MRS medium, which was then incubated at 30°C and in the dark for 72h [8]. The reference bacterial strain was *Staphylococcus aureus* ATCC 29213.

Purification and Identification of Lactic Acid Bacteria

The purification of the isolated bacteria was performed according to the method described [9]. Bacteria that showed a Gram positive stain and lacked catalase activity were separated for identification. They were submitted to a series of simple tests for preliminary identification: growth in MRS medium with addition of NaCl, growth at different temperatures and search of the homo- or hetero-fermenting type by the classical method [10, 11].

Demonstration of the Antagonistic Effect of Isolates Against *Staphylococcus aureus*

The lactobacillus species were inoculated in tubes containing 9 ml of MRS medium, which were incubated during 18h at 30°C.

Staphylococcus aureus ATCC 29213 was grown in Chapman broth and incubated for 18h at 37°C.

The surface of Petri dishes containing TSA medium was covered with a few milliliters of preliminary culture of *Staphylococcus aureus* strain and allowed to dry for 30 minutes at 37°C.

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Discs of blotting paper were soaked with the preliminary culture of lactic acid bacteria. They were left to dry and then placed on TSA media plates which, in turn, were allowed to dry for 30 minutes. Then they were left at 4°C for 4 hours to ensure the diffusion of the substances responsible for the interaction and finally incubated at 37°C for 24 hours.

The inhibition of the indicator strain results in the formation of clear zones around the disks, whose diameter (in mm) is measured from the periphery of the disc [12].

Effect of the Camel Milk Isolates on the Yoghourt Ferments

Two flasks with a volume of 110 ml of reconstituted milk, which had been sterilized in the autoclave at 120°C for 20 minutes, were inoculated with the strains of *Lactobacillus delbrueckii* subspecies *bulgaricus* and *Streptococcus thermophilus*, approximately 0.1023 g / l of milk [16]. A volume of 1 ml of camel milk isolates that showed strong inhibitory activity (approximately 5.87

log CFU / ml) against *Staphylococcus aureus* was added to one of the two bottles (bottle 1). Both flasks were incubated at 44°C for 4h.

Growth Kinetics

Sampling at increasing time intervals (1h, 2h, 3h, 4h) was followed by the preparation of decimal dilutions in Ringer solution. Then 0.1 ml of the appropriate dilution was plated on MRS medium for enumeration of *Lactobacillus delbrueckii* subspecies *bulgaricus* ferment and M17 for *Streptococcus thermophilus* ferment count.

After studying the characteristics that differentiate the species of lactic acid bacteria (isolates of camel milk and yoghurt ferment *Lactobacillus delbrueckii* subspecies *bulgaricus*) belonging to the same genus *Lactobacillus*, growth in the presence of 6.5% NaCl was shown specific for the isolate but not for the lactic ferment of the yoghurt Figure 1.

In order to eliminate any confusion, the count of camel milk isolate was performed on MRS medium with

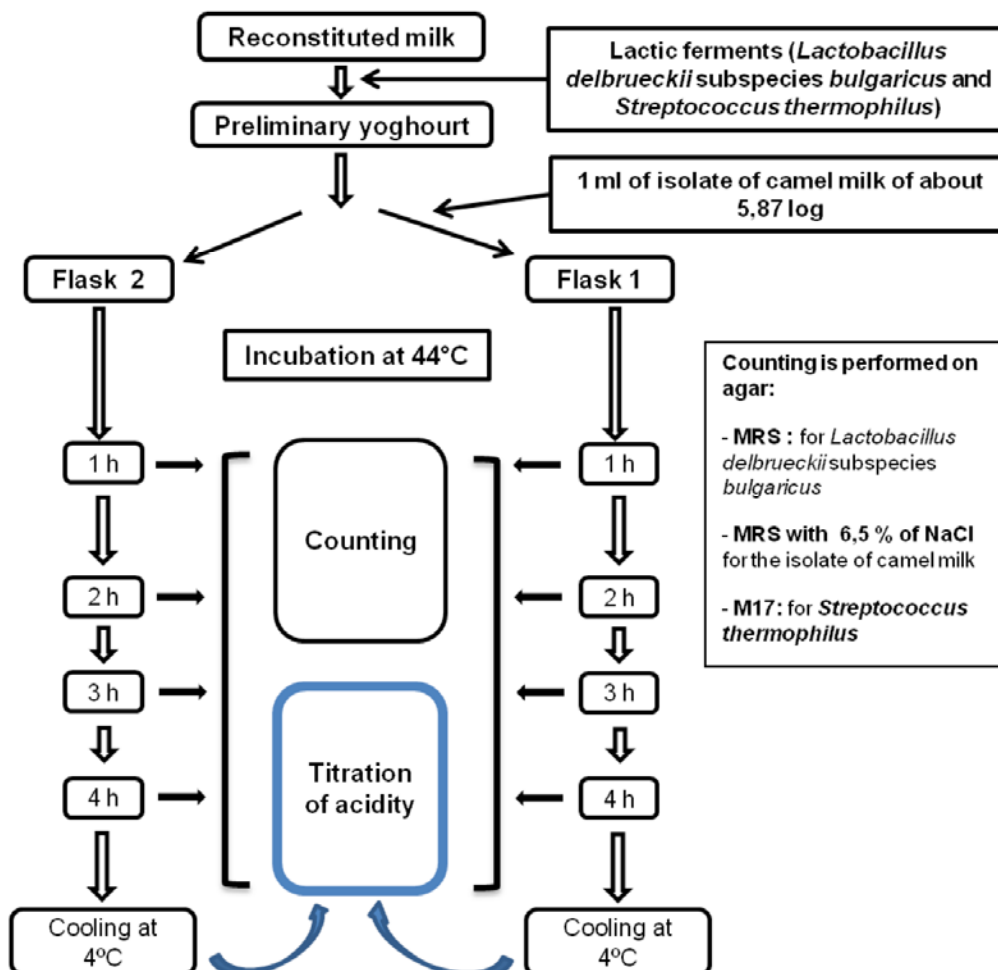


Figure 1: Experimental proceeding for the preparation of a yoghurt to which the isolate of camel milk.

addition of 6.5% NaCl. The acidity produced by pure strains was assessed by titration, sampling 10 ml from each bottle [13].

Effect of the Camel Milk Isolates on the Behavior of *Staphylococcus aureus* in the Yoghourt

Preliminary Culture Preparation

With each isolate of camel milk and *Staphylococcus aureus* were inoculated 10 ml of sterile skim milk. The preliminary cultures were prepared by incubation at 30°C until coagulation.

Preparation of the Preliminary Yoghourt

The preliminary yoghurt was prepared from reconstituted milk sterilized by autoclaving at 120°C for 20 minutes and inoculated with 0.01125 g / ml of lyophilized ferments.

The preliminary yoghurt was then dosed in 2 bottles:

- Bottle 1 was inoculated with 1 ml of *Staphylococcus aureus* from about 5.05 log CFU / ml.

- Bottle 2 was inoculated with 1 ml of *Staphylococcus aureus* from about 5.05 log CFU / ml and 1 ml of isolates of camel milk approximately 5.87 log CFU / ml.

Both flasks were incubated at the same time at 37°C for 5 h to achieve maximum growth of *Staphylococcus aureus*.

Samples were taken at regular intervals (1h, 2h, 3h, 4h, 5h) and decimal dilutions (10^{-1} to 10^{-4}) from the bottles were performed in order to make a count of *Staphylococcus aureus* on medium Chapman, which was then incubated at 37°C for 24 h.

Furthermore 10 ml extractions were performed to measure pH Figure 2.

RESULTS

Characterization of Isolates

The physiological and biochemical characteristics of the isolates are shown in the table below Figure 3.

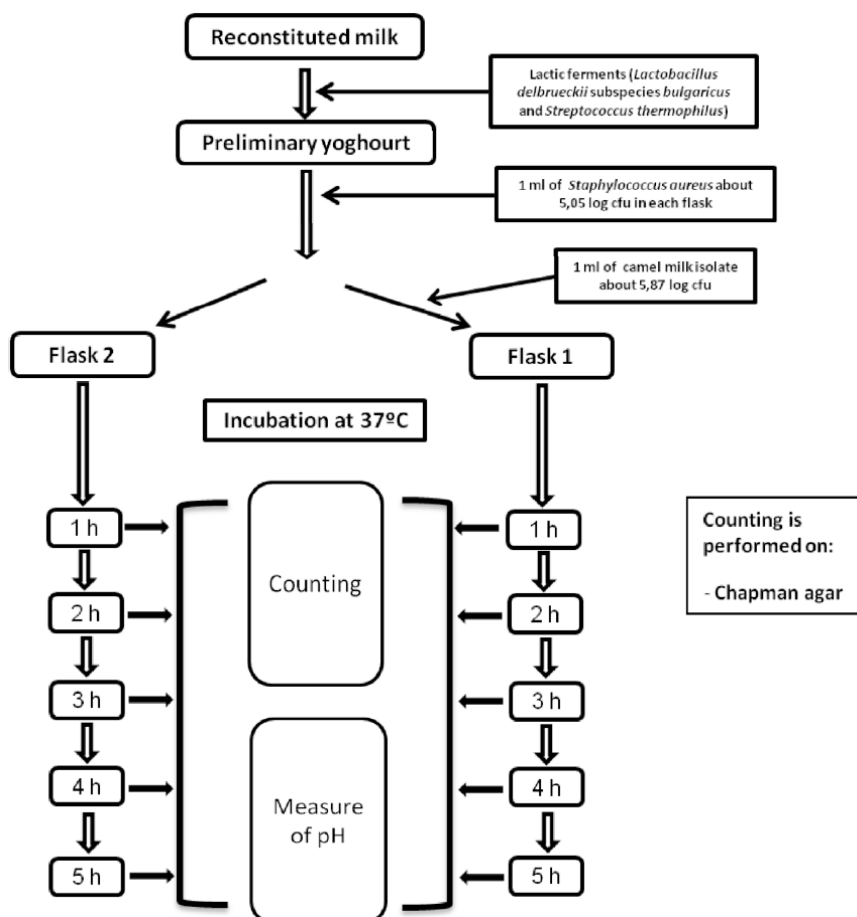


Figure 2: Diagram showing the preparation of a contaminated experimental yoghurt.

Characteristic	Isolate						
	Lc2	Lc3	Lc4	Lc7	Lc16	Lc17	Lc22
Gram	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-
Morphology	bacillus	bacillus	bacillus	bacillus	bacillus	bacillus	bacillus
CO ₂ on glucose	-	-	+	-	+	+	-
Growth at 45°C	+	+	+	+	+	+	-
Growth at 10°C	-	-	+	-	+	+	+
Growth with NaCl	2%	+	+	+	+	+	+
	3%	+	+	+	+	+	+
	6,5%	+	+	+	+	+	+
ADH	-	-	-	+	+	+	+
Esculine hydrolysis	+	-	-	-	-	-	+
Survival at 60°C / 30 minutes	+	+	+	+	-	+	+
Glucose	+	-	-	+	+/-	+	+/-
Raffinose	+	-	-	+	+	+	+
Lactose	+	+	-	+	+	+	+
Cellubiose	+	-	-	+	+	+	+
Mannitol	+	-	-	+	+	+	+
Arabinose	+	-	-	+	+	+	+
Xylose	-	-	-	+	-	+	+

Figure 3: Physiological and biochemical characteristics of the isolates from camel milk.

Among the 23 strains isolated from camel milk, 7 isolates were taken into account, all being Gram +, motionless, catalase negative and possessing bacillary form.

Strains LC4, LC16, Lc17 and Lc22 of *Lactobacillus* are classified within the group Betabacterium because they produce CO₂ on glycoside medium.

Strain LC4 shows growth at 10°C and is close to the species *Lactobacillus viridiscens*.

Strain LC16 develops at 45°C and ferments arabinose and raffinose, being close to the species *Lactobacillus buchneri*, while Lc17 hydrolyzes arginine and is close to the species *Lactobacillus fermentum*.

The streptobacterium group, which differs from the preceding one because there is no production of gas, is represented by strain Lc22, which grows at 10°C but not at 45°C.

Strain Lc22 ferments the group of sugars which were tested and is close to the species *Lactobacillus plantarum*.

Strains LC2, LC3 and LC7 grow at 45°C but not at 10°C and do not produce CO₂, and thus they are classified into the group Thermobacterium.

Strain Lc2 ferments the raffinose, hydrolyzes esculin and is close to *Lactobacillus acidophilus*, while strain Lc3 ferments lactose and is close to the species *Lactobacillus helveticus*.

Strain Lc77 hydrolyzes arginine and is close to the species *Lactobacillus delbrueckii* subsp *lactis*.

The large number of isolates which were obtained can be explained because the bacteria were incubated for 5, 7, 9 and 16 days.

Antagonistic Effect of Camel Milk Isolates on *Staphylococcus aureus*

The diameter registered for the inhibition zones varied from 07 mm (strain Lc2 showed the lowest activity) to 13 mm (the highest activity was recorded for strain Lc17) (Figure 4).

Strain (LC17), which has shown a strong inhibitory activity against *Staphylococcus aureus*, which is similar

Strain	TSA	
Lc 2	<i>Lactobacillus acidophilus</i>	07 mm
Lc 3	<i>Lactobacillus helveticus</i>	11 mm
Lc 4	<i>Lactobacillus viridiscens</i>	00 mm (no inhibition)
Lc 7	<i>Lactobacillus delbrueckii</i> subsp <i>lactis</i>	11 mm
Lc 16	<i>Lactobacillus buchneri</i>	11 mm
Lc 17	<i>Lactobacillus fermentum</i>	13 mm
Lc 22	<i>Lactobacillus plantarum</i>	10 mm

Figure 4: Diameter of the inhibition zones of the 7 isolates from the camel milk in front of *Staphylococcus aureus* by the method of diffusion on TSA medium.

to the species *Lactobacillus fermentum* (biochemical identification) is the one that was used in the following experiment.

Interactions between *Lactobacillus fermentum* and Yoghourt Ferments

Count of Yoghourt Ferment and *Lactobacillus fermentum* after 4 Hours in Mixed Culture

The growth of lactic acid bacteria was stationary after 4 hours of incubation both in camel milk (*Lactobacillus fermentum*) and in the yoghurt (*Lactobacillus fermentum* and *Lactobacillus delbrueckii* subspecies *bulgaricus* and *Streptococcus thermophilus*) Figure 5.

The number of lactic acid bacteria varied between 7.00 and 5.17 log CFU / ml.

The addition of *Lactobacillus fermentum* isolated from camel milk has no inhibitory effect against yoghurt ferments (*Lactobacillus delbrueckii* subspecies *bulgaricus* and *Streptococcus thermophilus*).

Biochemical Analysis

After 4 h of incubation the acidity increased significantly, reaching 58°D in the yoghurt which was inoculated with *Lactobacillus fermentum* Figure 6. After cooling, as a means for interrupting fermentation, the

acidity values of both types complied with AFNOR standards [16].

Effect of *Lactobacillus fermentum* on the Behavior of *Staphylococcus aureus* in the Yoghourt

It was not detected any antagonist effect on *Staphylococcus aureus* which was incorporated at about 5.05 cfu / ml in bottle 1 (yoghourt without *Lactobacillus fermentum*). By contrast, after addition of *Lactobacillus fermentum*, a decrease was detected after only 2 hours of incubation and after 4 hours there is a lack of growth of *Staphylococcus aureus*, which demonstrates the inhibitory effect of *Lactobacillus fermentum* Figure 7.

If a high burden of *Staphylococcus aureus* (> 5.5 log CFU / ml) is introduced, the effect of *Lactobacillus fermentum* is not noticeable.

Biochemical Analysis

The pH is stable in both types of yoghurt during the 2 hours of incubation, afterwards, a sharper decrease of pH is detected for both types of yoghurt Figure 8.

The final pH is a little high, since it has a value of 5.2 in comparison with the pH of common yoghurt which is 4.34. This is a consequence of the incubation

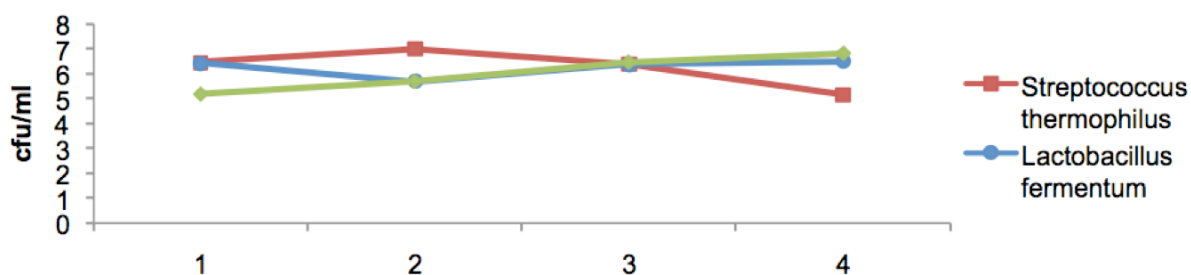


Figure 5: Growth of *Lactobacillus fermentum*, *Lactobacillus delbrueckii* subspecies *bulgaricus* and *Streptococcus thermophilus* in a mixed culture in the yoghurt.

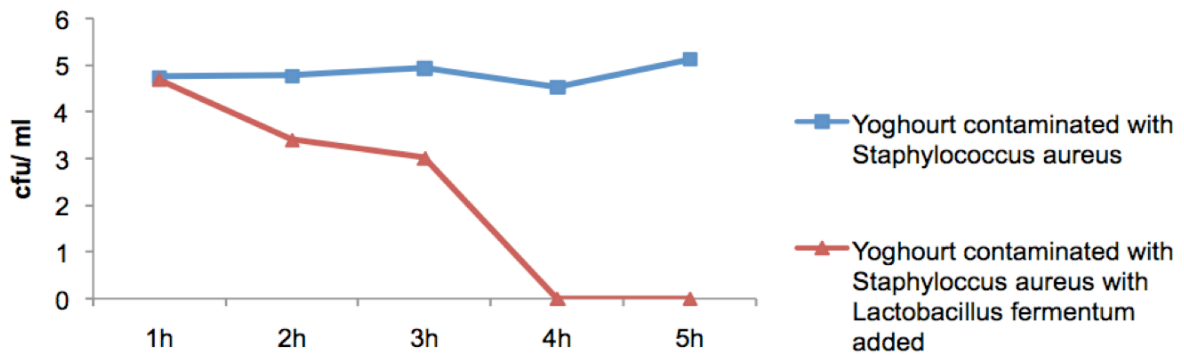


Figure 6: Growth of *Staphylococcus aureus* in an isolated and mixed culture with *Lactobacillus fermentum*.

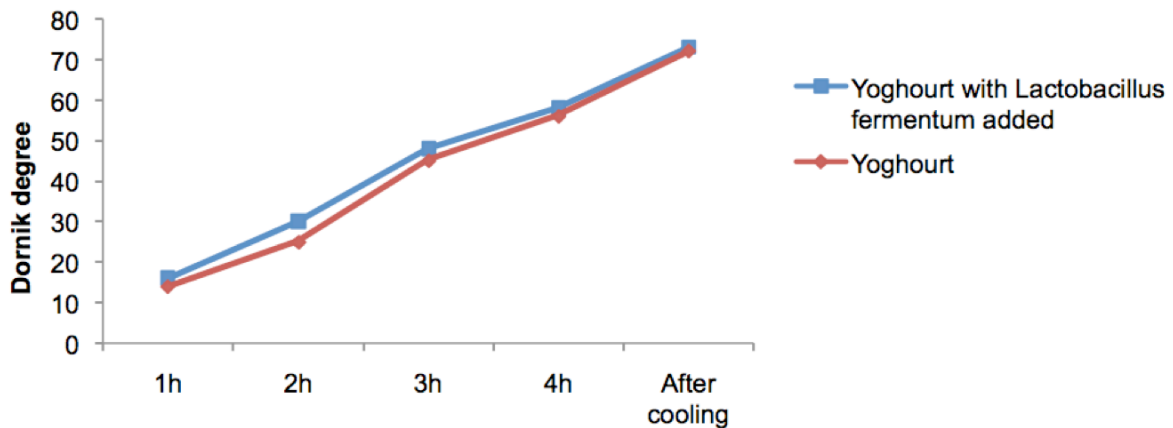


Figure 7: Evolution of the acidity during the maturation of both types of yoghurt expressed in Dornik degrees.

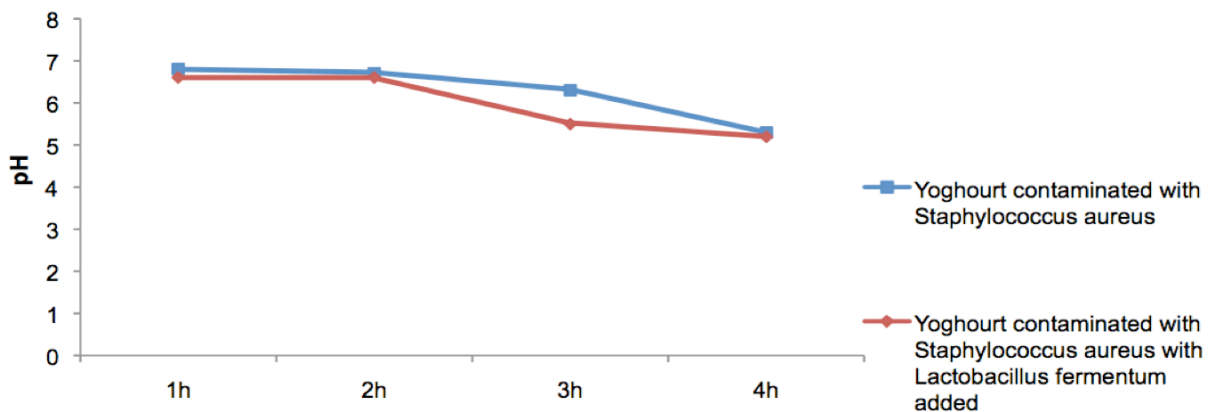


Figure 8: Evolution of pH during the maturation of the two types of yoghurt contaminated with *Staphylococcus aureus*.

temperature (37°C), which is lower than the temperature of maturation of the yoghurt (44 °C).

DISCUSSION

The seven isolates were all Gram +, motionless and catalase negative, confirming the data of Gunter *et al.* [17].

The fermentation profile of the seven *Lactobacillus* isolates was compared with the reference strains as exposed in Bergey's key [15]. Significant differences

were found. For example: *Lactobacillus fermentum* differs from the strain reference because of the fermentation of arabinose and mannitol.

The identified species *Lactobacillus plantarum* is only represented by the strain Lc22 while *Lactobacillus* strains isolated by Karam *et al.* [18] belong essentially to the species *plantarum*.

The addition of *Lactobacillus fermentum* isolated from camel milk has no inhibitory effect against yoghurt ferments (*Lactobacillus delbrueckii*

subspecies bulgaricus and *Streptococcus thermophilus*).

The acidity of the yoghurt where *Lactobacillus fermentum* was added meets AFNOR standards. The decrease in the number of living cells of *Staphylococcus aureus* from the 3rd hour of incubation in the presence of *Lactobacillus fermentum* shows the inhibitory effect of the latter. This result has been observed in the work of Mami *et al.*, 2010 in some species of *Lactobacillus* on *Staphylococcus aureus*. The results show a total lack of *Staphylococcus aureus* in the presence of *Lactobacillus fermentum* after only 4 hours of incubation in mixed cultures, whereas this was only observed by Mami *et al.*, 2010 after 72 hours.

The inhibitory effect of the *Lactobacillus fermentum* strain of *Staphylococcus aureus* at a concentration of up to 5.05 log CFU is nil.

In any case, the inhibition of *Staphylococcus aureus* is not caused by pH, because in the course of maturation the yoghurt contaminated with *Staphylococcus aureus* and without *Lactobacillus fermentum* has 3.90 log CFU of *Staphylococcus aureus* and the pH is 5.3 after 4 hours of incubation. Conversely, in the yoghurt contaminated with *Staphylococcus aureus* and to which *Lactobacillus fermentum* is added there is a total absence of *Staphylococcus aureus* with a similar value of pH, i.e. 5.2.

Inhibition of *Staphylococcus aureus* caused by the production of hydrogen peroxide must be excluded because *Staphylococcus aureus* has a catalase.

Competition for the amino acids in the milk medium cannot explain the inhibition of *Staphylococcus aureus*, because it grows in the milk without using aminoacids [14].

The capacity of *Lactobacillus fermentum* to produce bacteriocines putrescine at summer evoked by Mikelsaar *et al.*, so the inhibiting properties of *Lactobacillus fermentum* opposite *Staphylococcus aureus* rests on the production of the bacteriocines [19].

CONCLUSION

Camel milk contains lactic acid bacteria possessing interesting inhibitory potential against pathogens (*Staphylococcus aureus*). This study was based on a lactic strain represented by *Lactobacillus fermentum*.

Its bioproducteurs effect of molecules which inhibit pathogens leaves a great debate on the use of these molecules in the pharmaceutical treatment of certain pathology and also in the agroalimentary sector.

OUTSTRANDING PHRASES

The addition of *Lactobacillus fermentum* isolated from camel milk has no inhibitory effect on yoghurt ferments

The decrease in the number of living cells of *Staphylococcus aureus* from the 3rd hour of incubation in the presence of *Lactobacillus fermentum* shows the inhibitory effect of the latter.

Camel milk contains lactic acid bacteria possessing interesting inhibitory potential on pathogens (*Staphylococcus aureus*).

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