Behavior of *Staphylococcus aureus* in a cheese produced by local lactic acid bacteria

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Abstract: *Staphylococcus aureus* is a pathogenic bacterium that infects the milk and dairy products including cheese, causing food borne infections in humans. The objective is to scrutinize the growth and evolution of *S. Aureus* in cheese made with lactic acid bacteria isolated from local plants that are *Lactococcus lactis subsp. cremoris* and *Lactococcus lactis subsp.lactis* biovar *diacetylactis.* In the first part, the milk that was used in the manufacture of cheese was contaminated with *Staphylococcus aureus*, the antimicrobial activity was studied *vis-à-vis S. aureus* and the antagonistic effect of the latter against the lactic strains. In the second part, we performed counts of *lactococci* and *S. aureus* as the contaminated cheese in various stages of cheese production. From contaminated milk before manufacture of cheese, we have noticed a steady decline of lactic ferments after curdling and in parallel we have seen a decrease in the number of *S. aureus* during the early stages of production to increase again in the salting. The results of contaminated cheeses after their manufacture revealed a significant decrease in lactic strains and the pathogenic strain in both types of cheese after 24 and 72 h of their contamination.
Lactococci don't have inhibitory activity *vis-à-vis S. aureus*, and *S. aureus* did not inhibit lactic strains used in the manufacture of cheese. Thus, it preserves its contamination and poses a risk to human health.

Key words: *Staphylococcus aureus*, Contamination, cheese, local lactic acid bacteria.

Introduction

The lactic bacteria have presented a very crucial role in the manufacture of food fermented such as cheese for several centuries. The cheese is a complex medium made up mainly of water, coagulated proteins and milk fats in which, , the pH, the activity of water, the potential of oxydoreduction, the content of salt and the activity of the micro-organisms present in the leavens used, constitute a form of protection against the pathogenic ones. *Staphylococcus aureus* is a pathogenic bacterium which contaminates milk and the dairy products whose cheese, and for this reason it presents a major concern for animal and human health throughout the world. In certain contexts where lactic bacteria are the normal dominant microflore, as in cheese, *S. aureus* colonizes it sometimes and expresses the factors of virulence and produces a food poisoning. This study is devoted to manifest the interaction between the lactic acid bacteria and *S.aureus* and to show the capacities of inhibition which the lactic acid bacteria could have on the growth of S. *aureus* in the event of contamination milk which is used to produce cheese or its contamination after its production.

Materials and Methods

Material

Biological material

- **Milk**

Milk which has been used is a cow milk of the Montbeliard French race having 4 years old. It was selected following the selections carried out on several samples intended for the manufacture of cheese.

- **Rennet**

Commercial Rennet powder of forces 1/100.000 to 720 mg of Chymosine/100g. The powder of rennet is safely preserved from the light and moisture.

- **Lactic acid bacteria**

The lactic acid bacteria used are isolated starting from fermented plants (carrots, olives and cabbages) and identified at the local natural laboratory of Bioressources of the Faculty of Science of the university Hassiba Ben Bouali , Chlef, Algeria , they are *Lactococcus lactis subsp. cremoris* and *Lactococcus lactis subsp. lactis biovar diacetylactis.*

- ***Staphylococcus aureus***

The strain of *S.aureus* used, was isolated and identified at the local natural laboratory of Bioressources of the Faculty of Science of the university Hassiba Ben Bouali, Chlef, Algeria, starting from red Meat of cows.

Culture media

M17 broth and gelose (ref.: FABRI ms) For the preparation of the inoculum and the enumeration of the lactococci. For *Staphylococcus aureus,* we used the nutritive Broth (ref.: Pasteur institute of Algiers) for preparation of pre-culture, Giolitti Cantoni (ref.: Pasteur institute of Algiers) for enrichissement and Chapman medium (ref.: Pasteur institute of Algiers) for the enumeration of *Staphylococcus aureus*.
For the interaction between the lactococci and *S.aureus* Mueller-Hinton media (ref.: FABRI ms) is used.

Methods

The examination of the purity of the bacteria

The examination of the lactic bacteria is done by macroscopic and microscopic observations: the colouring of Gram and search for catalase are used. The scrutiny of *S.aureus* is made by macroscopic and microscopic observations, search of catalase and test of mannitol mobility.

For the transplanting of the lactic bacteria 1 mL of inoculum is ensemenced in 09 mL of milk. Homogenize and well sealed the tubes then incubated at 30 °C during 72 h.

For *Staphylococcus aureus* add 15 mL of a Potassium Tellurite solution to the medium of Giolitti Cantoni, and mix carefully. Carry aseptically 1 mL by dilution of *Staphylococcus aureus* in a sterile tube. Mix the medium and the inoculum.

The incubation is done with 37°C during 24 to 48 hours. Be presumed positive the tubes having transferred to black. To make sure that it is required a development of *Staphylococcus aureus,* these tubes will be the subject of a confirmation by isolation on Agar Chapman previously melted, casting petri dishes and thoroughly dried. Chapman boxes thus seeded will be incubated in their turn with 37°C during 24 to 48 hours. After this time to locate the suspect colonies with knowing the colonies of average size, smooth, brilliant, pigmented in yellow and equipped with a catalase and a coagulase.

Study of the antimicrobial effect of *Lactococcus sp.* on *Staphylococcus aureus*

***In vitro* study**

- Preparation of the pre-cultures

The lactic acid bacteria are ensemenced in tubes which contain 09 mL of M17 broth. The tubes are incubated during 24 h with 30 °C. *S.aureus* is inoculated into a tube containing 09 mL of nutritive broth; this tube is then incubated with 37° C during 24 h - 48 h.

Methods of interaction of *the L actococcus sp*. and *Staphylococcus aureus*

The interaction is tested according to the method of Tadesse *et al*. (2004) known as a disc method or carries germ, and that was made in two manners. Firstly, discs are impregnated *by S.aureus* and the lactic acid bacteria are cultured on agar, and secondly, the discs impregnated by the lactic acid bacteria and *S.aureus* ensemenced on agar.

Manufacture of cheese contaminated by *Staphylococcus aureus*

Raw materials

- **Preparation of milk**

14 g of dried milk is dissolved in 100 mL of distilled water, and then pasteurized in a Marie bath regulated with 75°C during 15 to 20 minutes. After that, milk is cooled at 37°C.

- **Preparation of the inoculum**

  Ensemence some colonies of lactic acid bacteria "*Lc. lactis subsp cremoris* and *Lc. lactis subsp lactis biovar diacetylactis* " in prepared milk (each strain in a bottle). Homogenize and well seal the bottles then incubate with 30°C during 24 hours.

- **Preparation of lactic ferments.**

Prepare o milk (dried milk 14g in 100 mL of distilled water); Take 2 mL of inoculum prepared in 100 mL of prepared milk then incubates at 30°C during 16 to 18 hours.

- **Preparation of the solution of rennet**

Dissolve 1g of rennet powder in 100 mL of distilled water and preserve at 4°C during one week in maximum.

- **Preparation of dilutions of the inoculum of *Staphylococcus aureus***

We aseptically take using a graduated pipette 1 mL of inoculum and introduce it into a sterile tube containing 09 mL of physiological water, this solution is regarded as dilution 10^{-1}, with the same method, one obtains dilutions 10^{-2},10^{-3}.

Stages of the manufacture of cheese

We conducted two ways: Prepared Cheese starting from milk contaminated by *S.aureus* and Cheese preparation then its contamination by *S.aureus*.

a/ Preparing cheese starting from contaminated milk by *S.aureus*

- **Curdling**
  - **Cheese of the lactic type**

- Pasteurize 1 liter of cow's milk in a Marie bath regulated in 75°C during 15 to 20 minutes;
- Cool the milk until the 30 - 37°C.
- Add 15 mL of lactic leaven of types *Lc. lactis subsp cremoris* and 15 mL of lactic leaven of type *Lc. lactis subsp lactis biovar diacetylactis;*
- Ensemence milk by 1 mL of dilution 10 -3 of the inoculum of Staphylococcus aureus;
- Homogenize and well seal the container;
- Leave the curdled milk at a temperature of 25°C during approximately 16 to 18 hours;

  - **- Cheese of the mixed type**

- Cheese curdling of the mixed type is identical to the first type of cheese, by adding 0,4 mL (1g/100 mL) with the solution of rennet for 1 L of milk;
- Ensemence milk by 1 mL of dilution 10^{-3} of the inoculum of *Staphylococcus aureus;*
- Homogenize and well seal the container;
- Leave the curdling milk at a temperature of 27°C during approximately 16 to 18 hours;

- **Draining**

- After the coagulation of milk, put the curd on a filter;

- Leave curd drained spontaneously during 24 hours;
- Recover the lactoserum;

- **moulding**

- The curd is put out of mould after 24 hours of draining;
- Make the 1 st reversal of the curd;
- After 12 hours, make the 2 nd reversal.

- **Salting**

- The curd is put in brine (6.5 % of NaCl) during 10 to 20 minutes;
- Follow-up of a final draining (24 hours at 18°C).

- **Refining**

-The refining and the conservation are 10 to 12 days at 14°C

b/ Contaminated cheese by *S.aureus* after its manufacture

Cheese of a lactic type and cheese of a mixed type with a concentration of 6.5% of NaCl are prepared with the same manner and they are contaminated after 10 days of conservation by *S.aureus.*

Enumeration of the lactic acid bacteria and *S.aureus*

The enumeration of the lactic flora and *S.aureus* is done along the principal stages of the manufactoring process of the cheese (initial Load of the inoculum, Curdling, Draining, moulding, draining, Salting, Refining, Cheese before contamination, Cheese after 24 h of contamination and 72 h of contamination). The enumerations are carried out for the two types of cheeses (lactic and mixed).

Results and discussion

Testing of the purity of the bacteria

a/ The lactococci

All the taken colonies starting from M17 are round or lenticular, with regular contours, white, opaque and smooth, indicating that there are the lactococci as was confirmed by Leveau and Bouix (1980), Guiraud (2003) and Badis *et al.* (2005).

After Gram colouration , the microscopic examination allowed us to notice the aspect of the cells and their mode of regrouping. Only the positive Gram bacteria are retained. The microscopic aspect of all the strains used is presented in the form of shells, as Guiraud (1998) indicates it. The lactococci ones do not have a catalase, which is confirmed by Guiraud (2003).

b/ *Staphylococcus aureus*

During the macroscopic identification of *S.aureus* on Chapman medium, the insulated colonies appear in the form spherical of gilded yellow color, with a regular contour with Catalase + and Mannitol-mobility + as it was indicated by Avril (1997).

The fermentation of the mannitol results in the appearance of a yellow color due to the acidification of the medium. The mobile bacteria produce disorders in the medium and the motionless bacteria persist meadows of the central puncture (Guiraud, 1998). Thus *S.aureus* ferments in anaerobiose the mannitol and it is motionless.

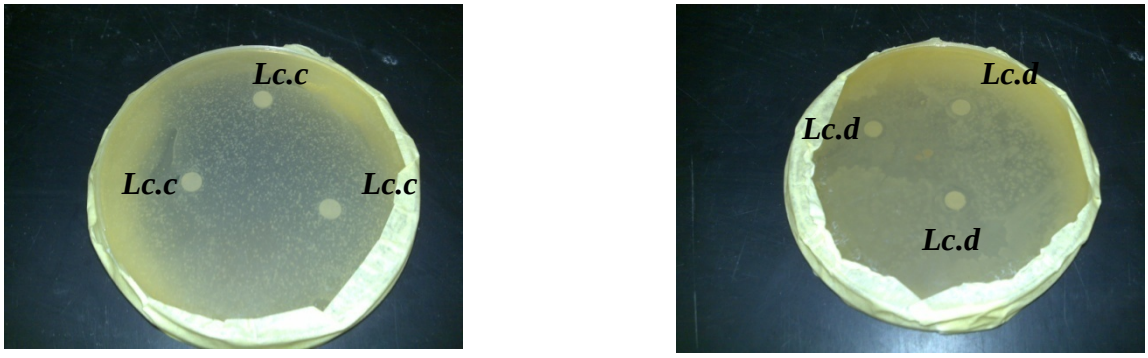
According to the results, we can deduce that it is about the *S.aureus* germ. These results are sustained by those of Baird Parker taken by Guiraud (1998).

Interaction of the *lactococcus sp.* and *staphylococcus aureus (in vitro)*

Inhibition of *S.aureus* by *lactococcus sp.*

According to the results obtained, no effect was noted, the pure cultures and the mixed cultures of lactococci do not have an inhibiting activity on S.aureus (figures 1and 2).

This result is not in agreement with the work of Schillinger and Lucke (1989) which revealed that the lactococci ones have an inhibiting activity, *in vitro*, of the strain of *S.aureus*. This difference is probably related to the origin of the local lactic acid bacteria which are isolated starting from the plants (carrots, cabbages and olive black) where the ecological niche is not the same one. In the same context, the work of Yuksekdag *et al.* (2004) manifest the capacity of lactococci to inhibit *S taphylococcus aureus in vitro*.



(A) (B)

Figure 1: Antibacterial activity of the pure cultures of *Lc. cremoris* (A) and *Lc. diacetylactis* (B) with *Staphylococcus aureus.*

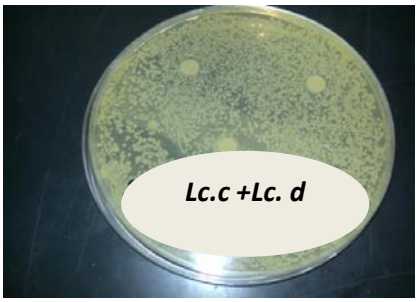
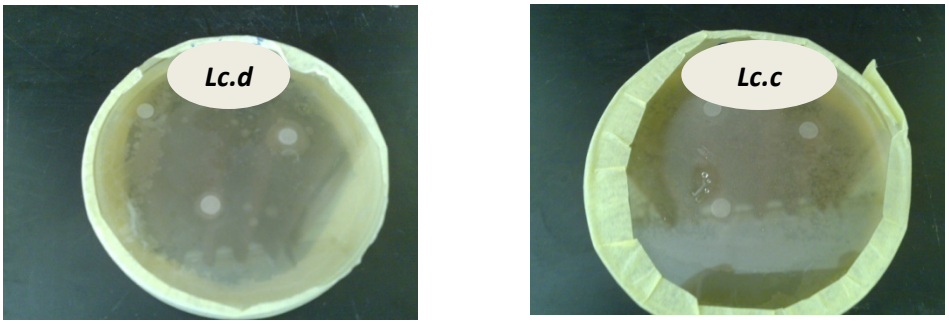


Figure 2: Antibacterial activity of the mixed cultures of *Lc. cremoris* + *Lc. diacetylactis* with *Staphylococcus aureus.*

Inhibition of *the lactococcus sp*. by *S.aureus*

When the discs were impregnated with *S.aureus* and the lactococci ones were spread out over the agar, we noted that there is no inhibition of lactococci by *S.aureus* (figures 3 and 4), which confirms that the lactococci ones used in the majority of the cases as probiotic against the pathogenic persons in charge for gastroenteritis are not inhibited by the latter what is confirmed by the former work of Luquet and Corrieu (2005) which showed that the intestinal lactic flora is a first line of defense which oppose to the microbes and the other infectious agents.



(A) (B)

Figure 3: antibacterial Activity of *Staphylococcus aureus vis-à-vis* of pure cultures of *Lc. cremoris* (A) and *Lc. diacetylactis* (B).

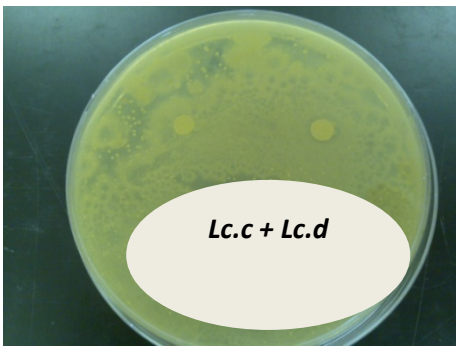


Figure 4: Antibacterial activity of *Staphylococcus aureus* on the mixed cultures of *Lc. cremoris* + *Lc. Diacetylactis*

Interaction of *the lactococcus sp.* and *staphylococcus aureus* in cheese

Prepared cheese starting from a milk contaminated by *S.aureus*

a/ The lactococci

Enumerations of the total lactic flora were carried out after each stage of development of the two types of cheese (lactic and mixed) contaminated by *S.aureus*. After the curdling of milk, the number of cells of the leavens is relatively high with 4.73×10^8 cells/mL for cheese of the lactic type and 3.2×10^8 cells/mL for cheese of the mixed type. The increase in the leavens is allotted to the substrates which are rich in nutriments for the lactic acid bacteria.

Subsequently and after the moulding and salting, the concentration of the leavens decreases relatively to reach respectively 1.8×10^7 cells/mL and 1.83×10^7 cells/mL for cheese of the lactic type and 2.75×10^7 cells/mL and 2.99×10^7 cells/mL for cheese of the mixed type. These results are in agreement with that of Kim et *al.* (1994) who observed a reduction in the population of lactococci in fresh and refined cheeses after salting. Undoubtedly, this is because of their sensitivity to salt, as well as the loss of a certain number of bacteria in the whey after draining. By this token, Mahaut *et al.* (2000) show that the content sodium chloride of milks of cheese dairy can influence the survival of the lactococci ones.

During refining, the number of cells of the leavens increases again to reach 7.06×10^7 cells/mL for cheese of the lactic type and 7.17×10^7 cells/mL for cheese of the mixed type after 4 days (figure 5). The bacterial concentration remains high after 8 days of refining for the two types of cheese. Then, the number falls again and this is due to the exhaustion of the medium in nutriments during the last days of refining. This result is in conformity with the one that has been found by Eck and Gillis (1997) which demonstrate that an 8 weeks refining to 12°C reduced little the population of lactic bacteria.

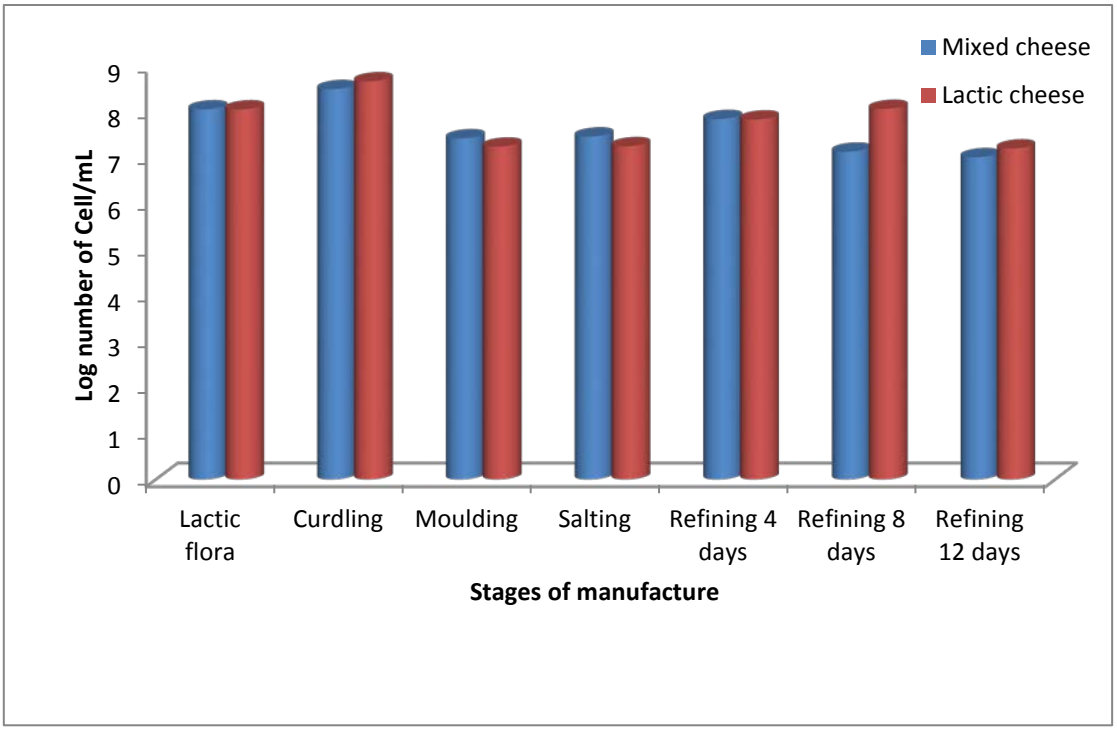


Figure 5: Enumeration of the cheese leavens during the stages of manufacture of cheese contaminated by *S. aureus*

b/ *Staphylococcus aureus*

The enumerations of the cheese ferments were carried out after each stage of development of the two types of cheese (lactic and mixed) contaminated by *S.aureus.* The initial load of *S.aureus* is 2.8 x 10^5 cells / mL in the two types of cheese. The results obtained indicate that the number of *S.aureus* falls considerably during the stages of curdling and the moulding for the two types of cheese manufactured and arrives respectively at 7.05 x 10^4 cells/mL and 6.25 x 10^4 cells/mL for cheese of the lactic type and 7.6 x 10^4 cells/mL and 4.5 x 10^4 cells/mL for cheese of the mixed type (figure 6), this reduction is allotted to the pH of milk. For this very reason, Leyral and Vierling (2007) showed that the lactic acid bacteria ferment lactose and acidify milk because of the massive production of lactic acid. Milk coagulates when the pH reached of the values less than 4.6 which is the isoelectric pH of milk.

Similarly, Hatreds and Hermon (1973) confirm that the inhibition of *S.aureus* by the lactic production of acid must take place after several hours in cheese. According to Gilliand and Speek (1974), antagonism towards *S.aureus* remains obvious when milk is maintained with a pH of 6.5. In the same context Meyrand *et al.* (1999) corroborates that the inhibition of *S. aureus* by the lactic acid bacteria is implemented at the time of the coagulation of milk.

The number of *S.aureus* increases again to reach a value of 6.31 x 10^4 cells/mL in cheese of the lactic type and 1.61 x 10^5 cells/mL in cheese of the mixed type during salting (figure 6). According to Han *et al.* (2005), *S.aureus* is an euryhaline bacterium which is able to multiply in medium of laboratory up to 12% of NaCl.

Throughout refining, we noted a reduction in the number of cells of *S.aureus* in the two types of cheese, which arrives at 1.24 x 10^4 cells/mL for cheese of the lactic type and 1.95 x 10^4 cells/mL for cheese of the mixed type, that can be explained by a proteolytic activity, a reduction in the activity of water (aw), an increase in the environmental temperature or an exhaustion of medium in nutriments.

Mayrand and Vernozy-Rozand (1999) displayed that the population of *Staphylococcus aureus* stabilizes itself or tends to decrease in particular during a long refining. But even after 12 days the number remains significant for a pathogenic bacterium such as *S.aureus* where their pathogenic character is directly related to the presence of toxins and its incidence on human health is only possible from starting from a strong

contamination: 10^6 Staph. / g of cheese, and even the regulation can be regarded as not satisfying a threshold more than 10*4* Staph. / g of cheese.

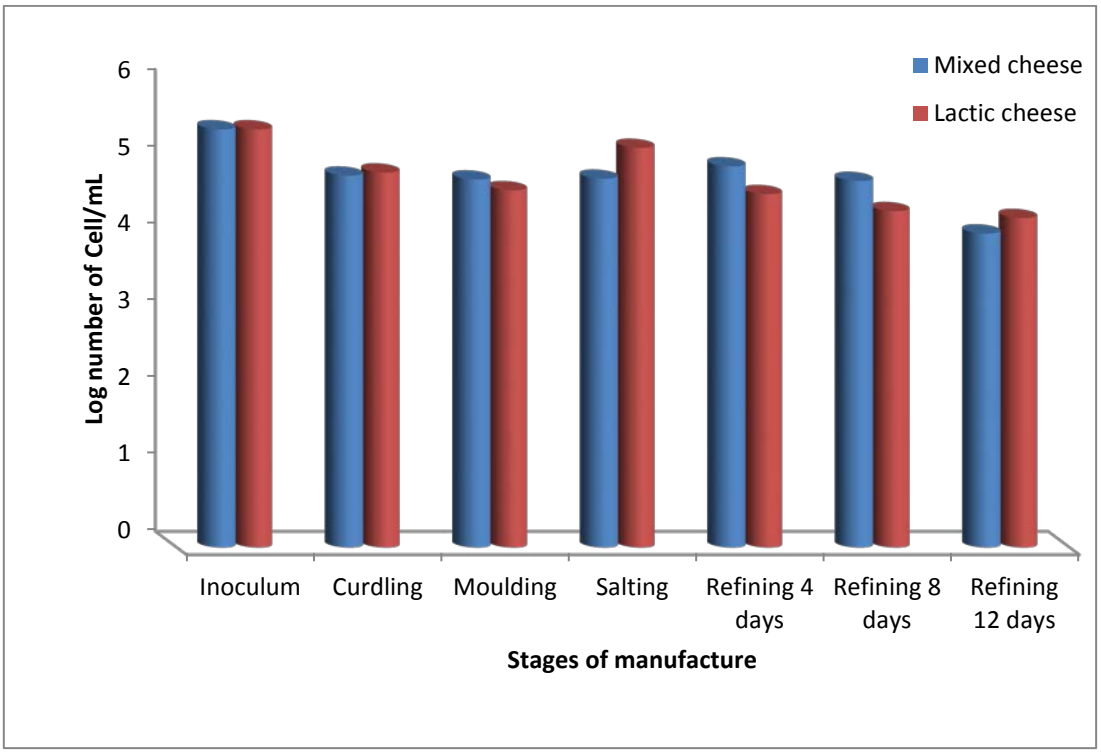


Figure 6: Enumeration of *S. aureus* during the stages of manufacture of cheese contaminated by S. *aureus.*

3.3.2. Cheese contaminated by *S.aureus* after its manufacture

We contaminated the two types of cheese by the *S.aureus* stock after their manufacture then, we carried out enumerations of the lactic strains and pathogenic strain after 24 and 72 hours.

a/ The lactococci

During the storage of cheese with 4°C, a reduction in the number of cells is recorded according to time (figure 7). The concentration reaches 3.36 x 10^7 cells/mL for cheese of the lactic type and 1.11 x 10^7 cells/mL for cheese of the mixed type after 10 days of storage. We noted that the load of the lactic flora decreases in the two types of cheese contaminated by the *S.aureus* strain. According to Bornarel *et al.* (2003) during marketing, the cheese is preserved at the cold, a temperature which has not to exceed 8°C, during 1 month or more. Under these conditions, the leavens of cheese do not multiply, but they, nevertheless, preserve a metabolic activity, the reduction is also allotted to the exhaustion of the medium in nutriments as the competitive effect between the strains.

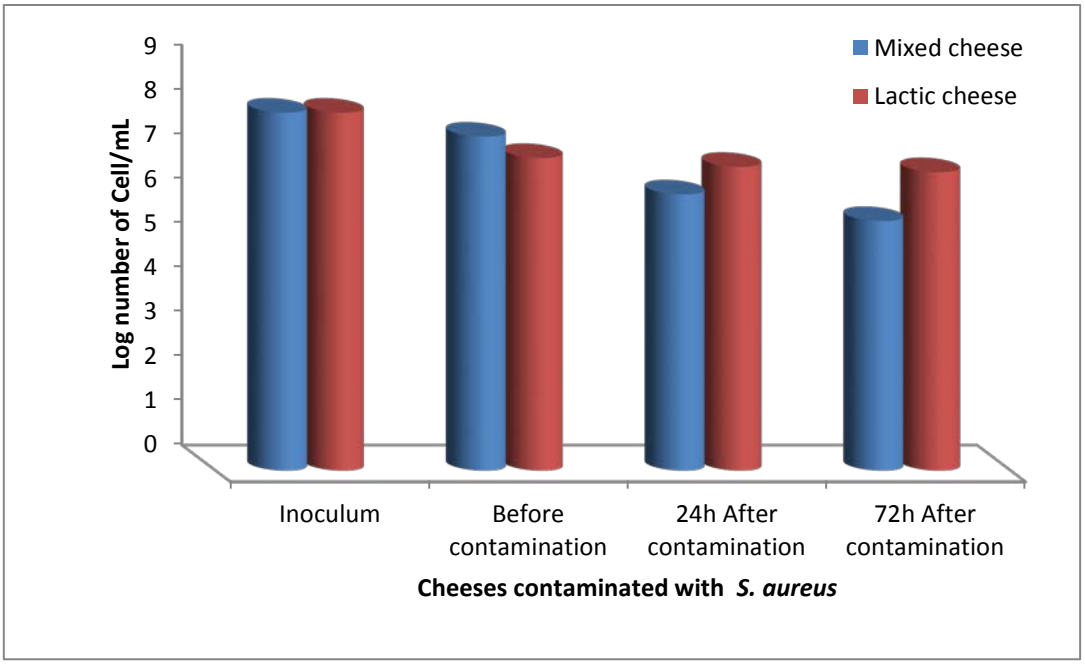


Figure 7: Enumeration of the lactic acid bacteria after the manufacture of contaminated cheese by *S. aureus.*

b/ *Staphylococcus aureus*

Following the results mentioned in figure 8, we observed a continuous reduction in a number of cells of *S.aureus* to arrive at a value of 0.86 x 10^4 cells/ mL in cheese of the lactic type and 0.15 x 10^4 cells / mL in cheese of the mixed type after 72h of their contaminations. According to Cathy (2006), the presence of the lactic acid bacteria in the cheese ecosystems generally operates a barrier effect on the establishment and the growth of *Staphylococcus aureus.* Along the same line of thought, Duquenne (2010) reports those technological agents and factors such as rennet, the leavens of acidification or refining, the mechanical and physical operations, the temperatures of manufacture and the conditions of refining can also influence the growth of *S. aureus.*

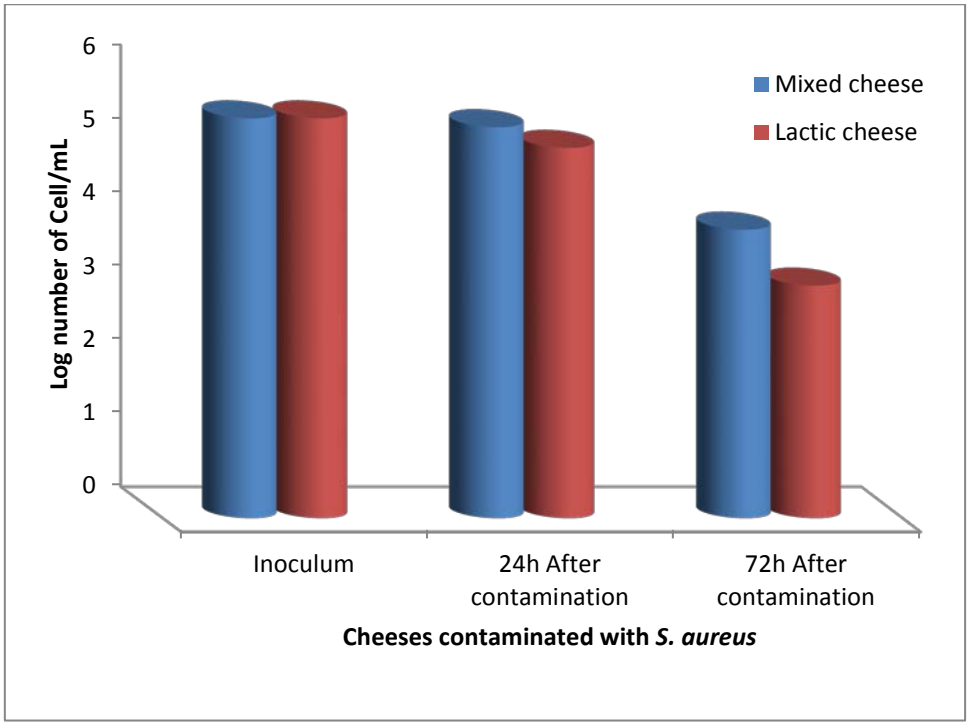


Figure 8: Enumeration of *S. aureus* in cheese contaminated after its manufacture.

Conclusions

The pure and the mixed cultures of *Lc. lactis subsp cremoris* and *Lc. lactis subsp lactis biovar diacetylactis* do not have an inhibiting activity with respect to *S.aureus* , of the same *S.aureus* did not inhibit lactic acid bacteria used *in vitro*.

The enumeration of lactococci and *S.aureus* during various stages of cheese-making manufacture (lactic and mixed) starting from contaminated milk being used to manufacture cheese showed a continuous reduction in the lactic leavens after curdling and in parallel the number of *S.aureus* fell during the first stages of manufacture to increase again during salting and that for both types of cheese.

The study of the evolution of the lactic strains and *S.aureus* in the two types of cheeses contaminated after their manufacture is characterized by a significant reduction in the lactic strains and pathogenic strain in the two types of cheese after 24 h and 72 h of their contaminations. Despite this, the cheese preserves its contamination *with S.aureus* in the course of time and remains a danger to human health.

Acknowledgement

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