Survive of *Clostridium perfringens* in a cheese produced by local lactic acid bacteria isolated from fermented olives

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Abstract

Cheese-making manufacture depends primarily on milk but also on the leavens necessary to its transformation. *Clostridium perfringens* is a pathogenic bacterium who contaminates meat products and dairy products, it is at the origin of human toxinfections. The aim of this study was to investigate the antimicrobial activity of local lactic acid bacteria strains on *Clostridium perfringens* on petri dishes and in cheese manufactured firstly by contaminated milk and secondaly after his production. Two strains of *lactococci* were used for the manufacture of cheese. They were *Lactococcus lactis subsp. cremoris* and *Lactococcus lactis subsp. diacetylactis* isolated from fermented olives. The results showed that *Clostridium perfringens* is sensitive to the effect of lactic acid bacteria on petri dishes and in cheese produced from a milk contaminated with the same strain. Lactic acid bacteria remain viable and preserve their probiotic effect with a significant number, whereas the pathogenic strain decreases over time.

Key words: *Clostridium perfringens, Lactococcus lactis subsp. cremoris, Lactococcus lactis subsp. diacetylactis,* local cheese, inhibition.

1. Introduction

Cheese-making manufacture depends primarily on milk but also on the leavens necessary to its transformation. The indigenous flora can come from the raw materials, the environment and various handling during the manufactoring process. It is of primary importance for the establishment of the organoleptic properties such as flavour, savour, texture and color, but also medical (ex: barrier effect, antimicrobic activities). Moreover, it can be made up at the same time of micro-organisms of technological interest, deterioration and the pathogenic ones.

Clostridium perfringens is a pathogenic bacterium, it has the characteristic to be demanding in growth promoters; it requires amino acids and vitamins for its growth. For this reason, only some food, in particular the meat products and in less degree the dairy products are contaminated and it is at the origin of human toxinfections which are caused by this germ. This study is devoted to manifest the interaction between the lactic acid bacteria isolated from fermented olives and *Clostridium perfringens* and to show the capacities of inhibition which the lactic acid bacteria could have on the growth of *Clostridium perfringens* in the event of contamination milk which is used to produce cheese or its contamination after its production.

2. Materials and Methods

2.1.Material

2.1.1. 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 is used. 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 olives and identified at the local natural Bioressources laboratory 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*.

- ***Clostridium perfringens***

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

2.1.2. Culture media

M17 broth and gelose (ref.: FABRI ms) for the preparation of the inoculum and the enumeration of the lactococci. For *Clostridium perfringens*, we used Columbia and VF media (ref.: Pasteur institute of Algiers). For the interaction between the lactococci and *Clostridium perfringens* Mueller-Hinton media (ref.: FABRI ms) is used.

2.2.Methods

2.2.1. The examination of the purity of the bacteria

The examination of the lactic bacteria and the scrutiny of *Clostridium perfringens* were done by macroscopic and microscopic observations, the colouring of Gram and search for catalase. 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 *Clostridium perfringens* carry aseptically 1 mL in 9 mL of nutritive broth. Mix the medium and the inoculum. The incubation is done at 37°C during 24 to 48 hours.

2.2.2. Study of the antimicrobial effect of *Lactococcus sp.* on *Clostridium perfringens* (*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. *Clostridium perfringens* is inoculated into a tube containing 09 mL of nutritive broth; this tube is then incubated at 37° C during 24 h - 48 h.

- **Methods of interaction of *the L actococcus sp*. and *Clostridium perfringens***

The interaction is tested according to the method of [1] known as a disc method or carries germ, and that was made in two manners:

- Firstly, discs are impregnated by *Clostridium perfringens* and the lactic acid bacteria are cultured on gelose;
- Secondly, the discs are impregnated by the lactic acid bacteria and *Clostridium perfringens* ensemenced on gelose.

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2.2.3. Manufacture of cheese contaminated by *Clostridium perfringens*

2.2.3.1. 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 sealing the bottles then incubate with 30°C during 24 hours.

- **Preparation of lactic ferments.**

Prepare milk (dried milk 14g in 100 mL of distilled water); Take 2 mL of inoculum prepared in 100 mL of 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 *Clostridium perfringens***

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}.

2.2.3.2. Stages of the manufacture of cheese

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

a/ Preparing cheese starting from contaminated milk by *Clostridium perfringens*

- **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 *Clostridium perfringens*;
- Homogenize and well sealing 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, the difference is only by adding 0,4 mL (1g/100 mL) with the solution of rennet for 1 L of milk;

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- Ensemence milk by 1 mL of dilution 10^{-3} of the inoculum of *Clostridium perfringens;*
- Homogenize and well sealing 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 *Clostridium perfringens* 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 *Clostridium perfringens* .

2.2.4. Enumeration of the lactic acid bacteria and *Clostridium perfringens*

The enumeration of the lactic flora and *Clostridium perfringens* 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).

3. Results and discussion

3.1. 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 [2-3-4]. 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 [5] indicates it. The lactococci ones do not have a catalase, which is confirmed by [3].

b/ *Clostridium perfringens*

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The colonies of *Clostridium perfringens* seem to appear in round forms and black color, They are Gram+, which appear under microscope as right bacilli with square ends only or in pairs. These properties were described by [6].

3.2.Interaction of the *lactococcus sp.* and *Clostridium perfringens (in vitro)*

3.2.1. Inhibition *of Clostridium perfringens* by *lactococcus sp.*

According to the results obtained, the pure culture of *Lactococcus lactis subsp, Cremoris* has inhibited *Clostridium perfringens* with 08 ± 0.14 mm of interaction diameter . On the other hand, *Lactococcus lactis subsp. diacetylactis* do not have an inhibiting activity on *Clostridium perfringens* (figure 1).

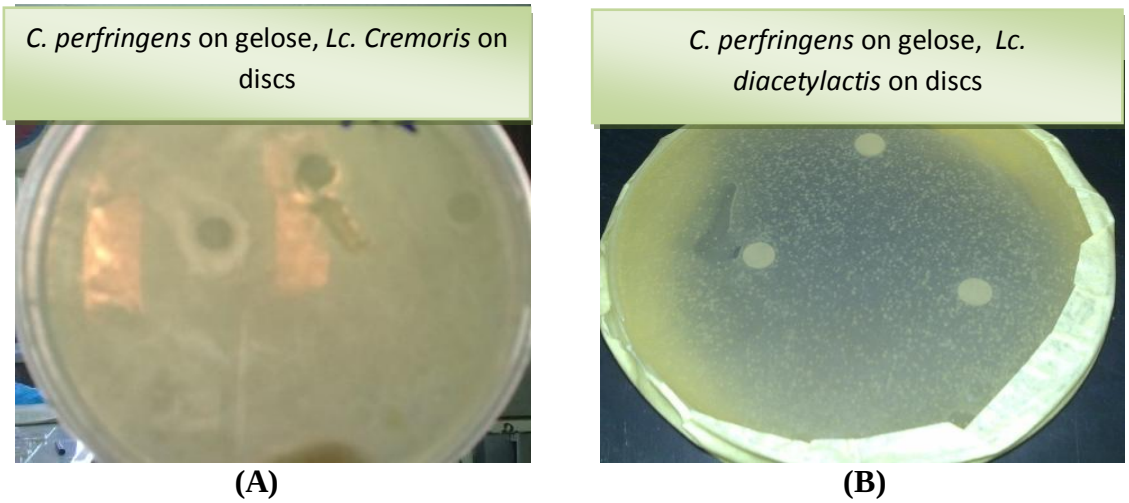
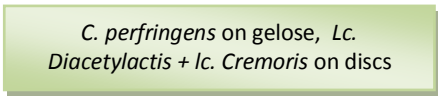


Figure 1: Antibacterial activity of the pure cultures of *Lc. cremoris* (A) and *Lc. diacetylactis* (B) with *Clostridium Perfringens.*

In mixed culture, the inhibitions were observed with a diameter of 09 ± 0.2 mm when the discs were impregnated by lactic stocks in combinations *(Lactococcus lactis subsp. cremoris + Lactococcus lactis subsp. diacetylactis) vis-à-vis* of *Clostridium perfringens* (figure 2).



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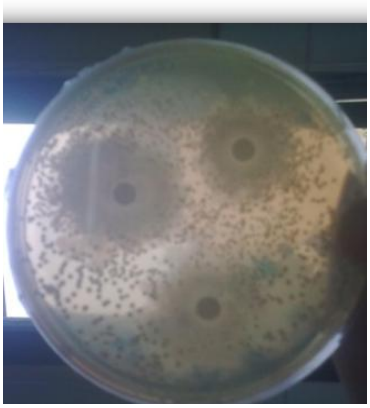

Figure 2: Antibacterial activity of the mixed cultures of *Lc. cremoris* + *Lc. diacetylactis* with *Clostridium perfringens.*

According to the these results, we can note that the two lactic acid bacteria, *Lactococcus lactis* subsp. *Lactis* biovar. *cremoris* and *Lactococcus lactis* subsp. *Lactis* biovar. *diacetylactis* , are active against the stocks of *Clostridium perfringens*. Similar results were found by [7-8-9] who revealed that purified bacteriocines which are named lacticine and lactococcine produced by *Lactococuss lactis* 481 are very active against the *Clostridium* genre.

3.2.2. Inhibition of *Lactococcus sp.* by *Clostridium perfringens*

Clostridium perfringens did not succeed to inhibit the two stocks *Lactococcus lactis subsp. cremoris* and *Lactococcus lactis subsp. diacetylactis* in pure culture.As a matter of fact , the inhibiting activity, after several tests, was always less than 2 mm of diameter.

No effect was noted too in mixed culture, 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. And this was confirmed by the former work of [10] which demonstrated that the intestinal lactic flora is a first line of defense which oppose to the microbes and the other infectious agents.

3.3.Interaction of *lactococcus sp.* and *Clostridium perfringens* in cheese

3.3.1. Prepared cheese starting from a milk contaminated by *Clostridium perfringens*

Enumerations were made to determine the loads of the lactic cultures as well as the pathogenic bacterium are used.

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 *Clostridium perfringens*.The lactic bacteria in mixed culture were present at initial rates of 1.15×10^8 cellules / mL in the two types of cheese (lactic and mixed). During curdling, we witnessed a reduction in the number of bacteria arriving respectively at $7{,}04 \times 10^6$ and 1.02×10^7 cells / mL in cheese of the lactic type and mixed type, this reduction is probably bound to the dispersion of the lactic bacteria between curd and Lactoserum. Once the latter is rejected, the residual load is weaker.

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Thereafter we attended the increase of the bacterial number during moulding, salting and beginning of refining to reach a value of 2.37 x10 ^{7} cells /mL for cheese of the lactic type and 1.89 x10^{7} cells / mL in cheese of the mixed type .These results are in agreement with that of [11] 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,. By this token, [12] 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 5.42 x 10^{6} and 3.09 x 10^{6}cells / mL respectively for cheese of the lactic type and cheese of the mixed type, that is allotted to the impoverishment of the medium to nutriment necessary for Lactococci, moreover the acidification whose acid pH avoids the development of the micro-organisms or involves a reduction of their number [13].

- A difference in a number of bacterium is announced between the two types of cheese, the little difference for cheese of the mixed type is allotted to the enzymatic action of coagulation by rennet because she reinforced curdling by decreasing the lactic activity slightly. Following figure (3) illustrates the results obtained:

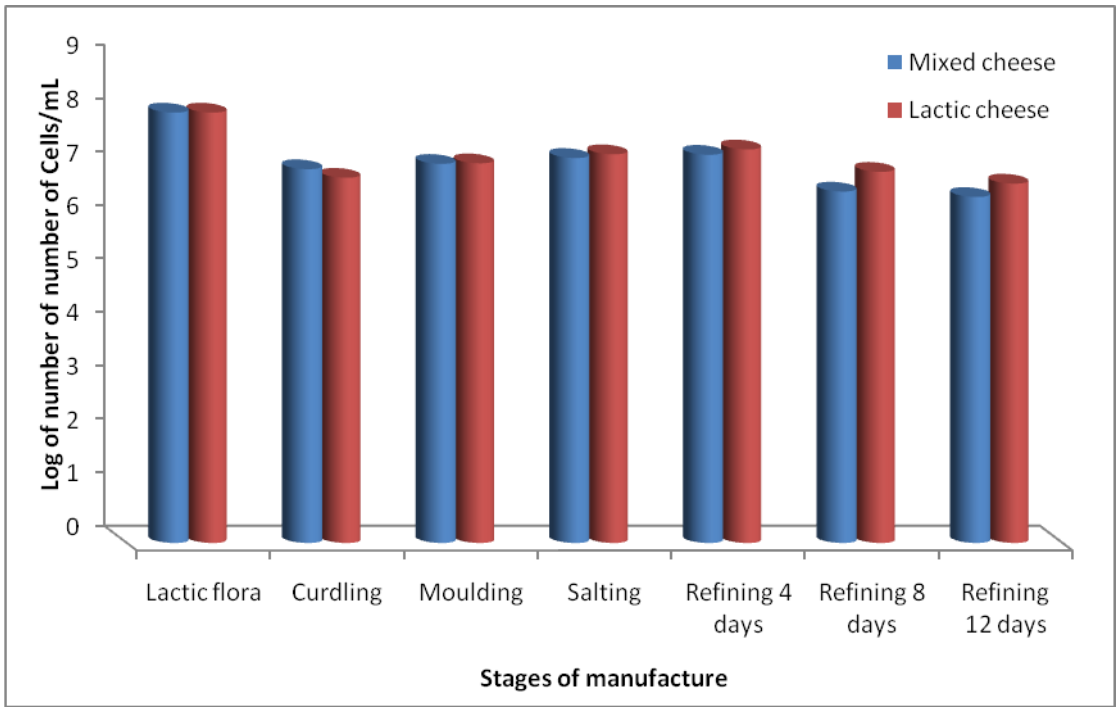

Figure 3: Enumeration of the cheese leavens during the stages of manufacture of cheese manufactured by milk contaminated by *Clostridium perfringens*.

b/ *Clostridium perfringens*

For the pathogenic stock, the inoculum by which we contaminated milk intended to manufacture the cheese was of 2.6 X 10 ^{6} cells / mL.

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During the first operation which is the curdling, we attended a little increase in the number of *Clostridium perfringens*, which arrives at 5.48 x 10^6 cells / mL for cheese of the lactic type and 3 x 10^6 cells / mL for cheese of the mixed type, and that is attributed at the favorable medium for the growth of this stock. But beyond that we attended a decrease in the number, especially following salting to reach a value of 1.4 x 10^5 cells / mL in cheese of the lactic type, and 2.6 x 10^5 cells / mL in cheese of the mixed type (figure 4), that attributed changes to the composition of the substrate as to the competitive effect with the lactic bacteria on nutriments. According to [14] and [15], the *Lactococci* are able to inhibit *Clostridium perfringens* considering the production of bacteriocines and lactic acid.

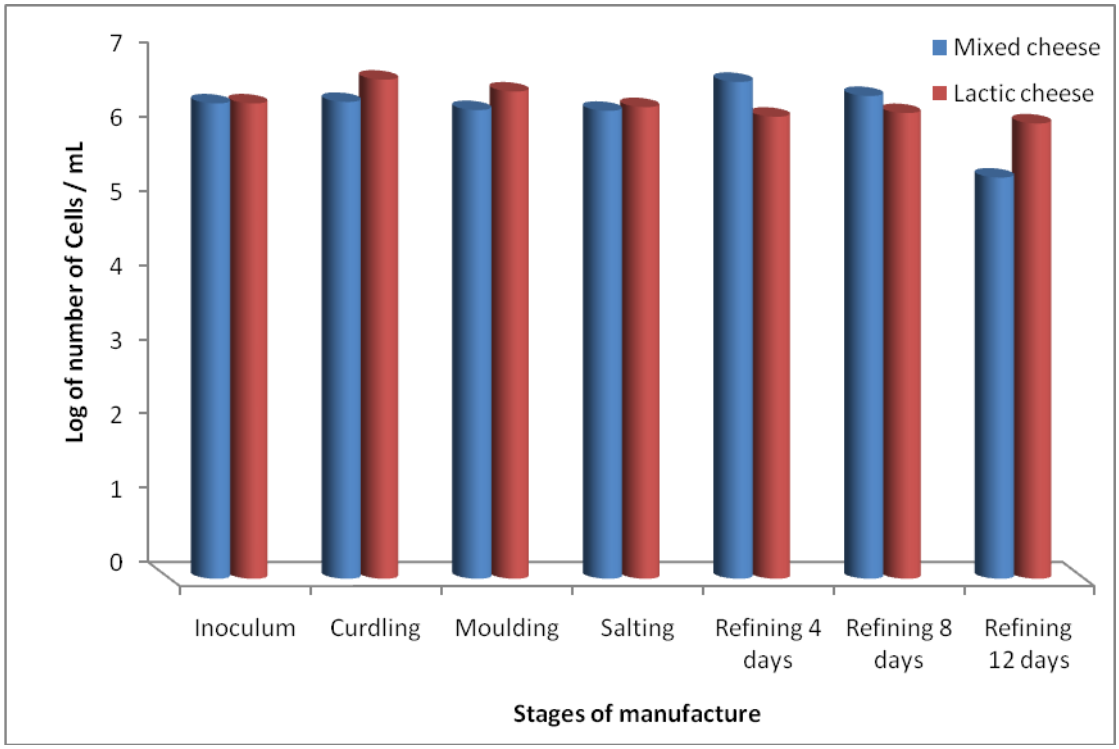

Figure 4: Enumeration of *Clostridium perfringens* during the stages of manufacture of cheese manufactured by milk contaminated by *Clostridium perfringens*

3.1.1. Cheese contaminated by *Clostridium perfringens* after its manufacture

We contaminated the two types of cheese by *Clostridium perfringens* after their manufacture then, we carried out enumerations of the lactic strains and pathogenic strain after 24 and 72 hours. In this case when the cheese was contaminated after its manufacture by *Clostridium perfringens*, we noticed that the number of *Lc. lactis subsp cremoris* and *Lc. lactis subsp lactis biovar diacetylactis* is similar to the preceding case considering that the processes are the same (figure 5), but for *Clostridium perfringens*, the number of cells has increased after 24h of contamination, it was 2.63x10^6 and 3.60x10^6 cells /mL respectively in cheese of the lactic type and mixed type (figure 6).

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Indeed, [13] confirmed that the level of population is variable according to the number of *Clostridium perfringens* in cheese, and this is in relation to the lactic acid bacteria during manufacture, the pH, the aw, the concentrations of NaCl and size of cheese.

After 72h, a considerable reduction in the bactrian load is noticed, for example it was 1.21x10 ^{6}and 2.32 x10 ^{6} cells/mL respectively in cheese of the lactic type and mixed type, and that is allotted to the number of lactic acid bacteria, which carried on a more significant inhibiting activity, from where the fall of the number of *Clostridium perfringens*. For this, and according to [16], the intoxications due to *Clostridium perfringens* are seldom in the technology of cheese-making , the parameters of acidification, the water content and the content of salt does not allow the growth of the vegetative cells of this bacterium and the germination of their spores.

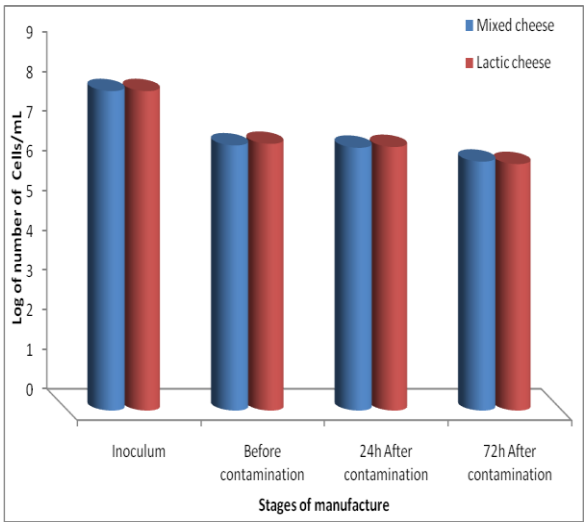

Figure 5: Enumeration of the lactic acid bacteria in cheese contaminated after its manufacture by *Clostridium perfringens.*

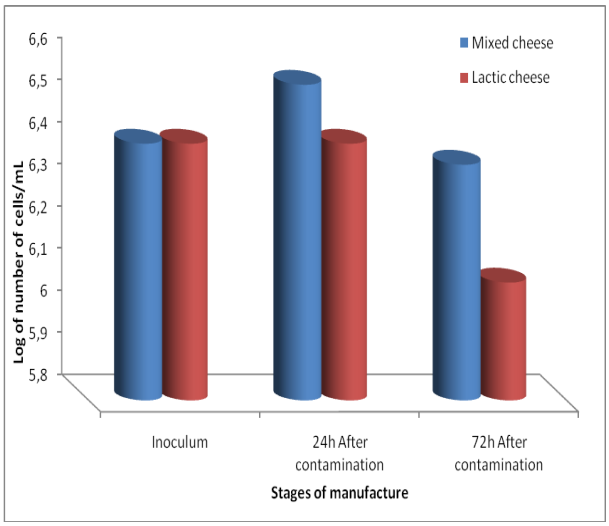

Figure 6: Enumeration of *Clostridium perfringens* after the manufacture of contaminated cheese

Conclusions

This work aimed primarily at studying the behavior of the pathogenic bacterium *Clostridium perfringens* in cheese, and the investigation of the effect of the lactic acid bacteria isolated from fermented olives. The study of the antimicrobial effect on petri dishes revealed that *Clostridium perfringens* is sensitive to the effect of *Lactococcus lactis subsp. Cremoris* and *Lactococcus lactis subsp. diacetylactis* in mixed culture, the diameter of inhibition arrived at 09 mm, on the other hand *Clostridium perfringens* did not succeed in inhibiting the lactic acid bacteria. The cheese manufactured with a milk contaminated by 2.6 x 10 ^{6} cells/mL of *Clostridium perfringens*, preserved a significant load , it started to decrease only in phase of refining.

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When the contamination by *Clostridium perfringens* is carried out after manufacture of cheese, the pathogenic bacterium was inhibited by the lactic acid bacteria after 72 hours of its contamination, the latters are known for their capacity to produce at the time of their growth active compounds like the organic acids, the hydrogen peroxide and o the natural substances of protein nature endowed with an antagonistic activity against a great number of pathogenic germs. The loads of the lactic acid bacteria were appreciable, and their reduction was announced only during refining. The presence of pathogenic bacteria in a food will have to be examined from the point of view of analysis of the risk incurred by the consumer with respect to these micro-organisms. The control of these pathogenic bacteria in the milk and the dairy products require the installation of systems of control and monitoring.

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