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Control of *Staphylococcus aureus* in Meat System by *in situ* and *ex situ* Bacteriocins from *Lactobacillus sakei* and *Pediococcus* spp.

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Abstract**—**The present study consisted of an applied test in meat system to assess the effectiveness of three bio agents bacteriocin-producing strains: L_{m24}: *Lactobacillus sakei*, $L_{m14}$and L_{m25}: *Pediococcus* spp. Two tests were carried out: The *ex situ* test was intended for three batches added with crude bacteriocin solutions at 12.48 AU/ml for L_{m25} and 8.4 AU/ml for L_{m14} and L_{m24}. However, the *in situ* one consisted of four batches; three of them inoculated with one bacteriocinogenic L_{m25}, L_{m14}, L_{m24}, respectively. The fourth one was used in mixture: $L_{m14+m24}$ at approximately of 10^7 CFU/ml. The two used tests were done in the presence of the pathogen *St. aureus ATCC 6538*, as a test strain at 10^3 CFU/ml. Another batch served as a positive or a negative control was used too. The incubation was performed at 7°C. Total viable counts, staphylococci and lactic acid bacteria, at the beginning and at selected times with interval of three days were enumerated. Physico-chemical determinations (except for *in situ* test): pH, dry mater, sugars, fat and total protein, at the beginning and at end of the experiment, were done, according to the international norms. Our results confirmed the *ex situ* effectiveness. Furthermore, the batches affected negatively the total microbial load over the incubation days, and showed a significant regression in staphylococcal load at day seven, for L_{m14}, L_{m24}, and L_{m25} of 0.73, 2.11, and 2.4 log units. It should be noticed that, at the last day of culture, staphylococcal load was nil for the three batches. In the in situ test, the cultures displayed less inhibitory attitude and recorded a decrease in staphylococcal load, for L_{m14}, L_{m24}, L_{m25}, $L_{m14+m24}$ of 0.73, 0.20, 0.86, 0.032 log units. Therefore, physicochemical analysis for L_{m14}, L_{m24}, L_{m25}, $L_{m14+m24}$ showed an increase in pH from 5.50 to 5.77, 6.18, 5.96, 7.22, a decrease in dry mater from 7.30% to 7.05%, 6.87%, 6.32%, 6.00%.This result reflects the decrease in fat ranging from 1.53% to 1.49%, 1.07%, 0.99%, 0.87%; and total protein from 6.18% to 5.25%, 5.56%, 5.37%, 5.5%. This study suggests that the use of selected strains as L_{m25} could lead to the best results and would help in preserving and extending the shelf life of lamb meat.

Keywords**—**Biocontrol, *in situ* and *ex situ*, meat system, *St. aureus*, *Lactobacillus sakei, Pediococcus* spp.

I. Introduction

LACTIC acid bacteria have been isolated and characterized from a variety of meat products, from the most common as red meat [1], [2], to the less common as dry salami [3]. Those potency bacteriocenogenic were largely isolated from various microbiota as seafood [4], chilled and processed meat and meat products [4]-[8], fermented meat [9], dry cured sausages [10]. Other works have been cited in the literature focused on research, identification and characterization of bacteriocins produced by lactic acid bacteria as well as those isolated from other foodstuffs [11]. Many of these works have beenabout the development of bacteriocinogenic cultures for food applications [12]. It is well known that lactic acid bacteria are harmless for human, which has led to the recognition of their GRAS status "Generally Recognized As Safe" [13]-[15]. Hence, these bacteria play a variety of healthful roles as probiotic, protective or starter cultures [1], [12], [14], [16], [17]. One of the most important purposes is to improve safety and stability brought by inactivation of pathogens and inhibition of spoilage bacteria, as well to reduce the application of chemical preservatives for meats. It would be more interesting to add protective cultures or their metabolite compounds namely bacteriocins [12], [16].

Bacteriocins are a heterogenous group of antibacterial proteins that vary in spectrum of activity, mode of action, molecular weight, genetic origin, and biochemical properties [15], [18], [19]. Most of them are cationic, hydrophobic or amphiphilic composed of 20-60 amino acid residues [20]. Through biochemical and genetic characterization, four major classes exist: (I) lantibiotics, (II) small heat stable peptides (III) large heat-labile proteins (IV) and complex bacteriocins [21]. The majority of them falls into classes I and II [22].The latest is among the most likely to be used in food applications, due to their specificity [14]. Several properties make them suitable for biopreservation since they are safe, neither active, or toxic on eukaryotic cells, and inactivated by digestive proteases [12]. As well, bacteriocins can be added as concentrated and not purified preparations or they could be produced *in situ* by bacteriocinogenic protective cultures. Moreover, immobilized bacteriocin forms can be used too for the development of active packaging [12], [22], [23].

Fresh meat constitutes an appropriate environment for the development of several types of microorganisms of endogenous or exogenous origin due to their composition and physico-chemical characteristics such as; their oxygen availability, water and hydrogen ion activities, associated with environmental factors. In low temperatures and under aerobic conditions, psychrophilic bacteria, particularly Gram-negative, will be involved in undesirable proliferation. While, in anaerobic conditions, anaerobes and facultative anaerobes bacteria tend to grow fast [22], [24]-[26]. Among these

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microorganisms, we note different pathogenic bacteria such as *St. aureus.* This pathogen may induce in meat lipolysis and proteolysis reactions that lead to some chemical changes [27]. As well known, *St. aureus* is a major pathogen responsible of foodborne intoxication and considered to be one of the most frequently occurring staphylococcal gastroenteritis worldwide [26], [28]. The symptoms occur within 2-4h.They are related to the potency and amounts of enterotoxin ingested and to the individual's resistance [26].

Recent studies are increasingly directed towards biocontrol of food-borne pathogens using protective lactic acid bacteria, or their bacteriocins [28]-[32]. This study was aimed to show off biocontrol of *St. aureus* in meat system and to assess the antimicrobial effectiveness by *in situ* and *ex situ* bacteriocins from autochthonous *Lactobacillus sakei* and *Pediococcus* spp. in order to show their future application.

II. Material and Methods

A. Bacterial Strains and Growth Media

Lactobacillus sakei (L_{m24}) and two lactic belonging to *Pediococcus* spp. (L_{m14} and L_{m25}) were used as bacteriocins producer. The cultures collection was maintained frozen at -18°C in 20% glycerol [33]. After that, working cultures were put as slants agar on MRS (De Man., Rogosa & Sharpe, Fluka®). The enterotoxin producer strain *St. aureus ATCC 6538* provided by the "L.N.C.P.P" (Laboratoire National de Control de Produits Pharmaceuriques, Oran).The pure culture was used as a challenge strain which has been regenerated in TSB (Trypticase Soy Broth, Difco®) at 37 °C during 24 h [34].

B. Crude Bacteriocin Preparation

The crude bacteriocin solutions were obtained after centrifuging at 6000rpm i.e. 3461×g for 20min [6] from overnight lactic cultures at 37°C for 24h in MRS medium, then were subject to heat treatment of 10min after which a sudden cooling of 4°C served to eliminate vegetative forms [35]. After that crude bacteriocins were tested for activity against the challenge strain using the well diffusion assay [36]. Dilution that resulted in the disappearance of the inhibition zone corresponded to the minimum inhibitory dose of pathogen growth indicator [37], [38].

C. Meat Model System

Fresh lamb meat was purchased from the retail in Saida city (western Algeria) to prepare the meat system. The outer surface of meat pieces was sterilized by immersion in 95 % (V/V) ethanol and then burning the residual ethanol on the muscle surface [39], then passes aseptically through a mechanical chopper to preparing a suspension with a saline-peptone water (NaCl 8.5g/L; bactopeptone 1g/L) in a ratio of 1:2 (W/V). The resulting suspension was then mixed in Stomacher (LAB BLINDER© 400) for 3 minutes to ensure a good homogenization. The sterility of the meat system was checked out by determining the absence of total viable counts using PCA agar (Plate Count Agar, Pronadisa™) incubated at 30ºC for 48h.

D. Meat ex situ and in situ Inoculation

Two trials were carried out to investigate the effectiveness of bacteriocin produced *ex situ* and *in situ*. Trial *ex situ* consisted of three batches in the presence of the pathogen *St. aureus ATCC 6538*, as a challenge strain, at approximately 10^3UFC/ml with the separate addition of crude bacteriocin solutions at 12.48 AU/ml and 8.4 AU/ml for L_{m25} and L_{m14}, L_{m24} at final culture volume of 20 ml by flasks. Trial *in situ* consisted of four batches in the presence of the pathogen *St. aureus ATCC 6538*, as a challenge strain, at approximately 10^3 UFC/ml. Three of them inoculated with the bacteriocinogenic L_{m14}, $L_{m24,}$ L_{m25} and the fourth in mixture: $L_{m14+m24}$ at approximately 10^7 UFC/ml. A positive control batch, at the same charge and a negative control without pathogen, were made. Culture experiments were carried out at +7°C for 9 days.

E. Microbiological Enumerations

Amounts of 1ml from culture batches were removed at three days intervals spaced (0, 3, 6 and 9) during the storage. A 1: 10 dilutions were made, homogenized for 1 min using stomacher and serial decimal dilutions were then prepared, using saline-peptone water. Aliquots were enumerated by spread or pour plate using appropriate dilutions as: total viable counts, *St. aureus*, and lactic acid bacteria. On the respective selective media: PCA agar incubated at 30ºC for 48h, BP agar (Baird-Parker, Pronadisa™) incubated at 37ºC for 48h and MRS agar incubated at 30ºC for 48h. The norms: ISO 2293, ISO 6888-1 and ISO 15214 respectively were used. Incubations were carried out in incubators (BINDER© B 115).

F. Physico-Chemical Determinations

The physico-chemical experiments were performed at the beginning and at the final of *in situ* test. The pH of sufficient aliquot to immerse the combined electrode of electronic pH meter (HANNA©210).The dray mater was determined based upon the principle of loss of weight by drying at 103°C to constant weight in the oven (BINDER© 14 D).The total fat amount was determined using Soxhlet apparatus including acid digestion with hydrochloric acid 4 N, filtration, drying, extraction with diethyl ether and finally, after separation with Rotavapor (BÜCHI© RE111) fat will be weighed. However, proteins were estimated by Kjeldahl method which consists on mineralization in boiling sulfuric acid with a catalyst, then distillation (BÜCHI© 11071 distillation units) of liberated ammonia into a boric acid solution, and finally titration with sulfuric acid to colorimetric point complete this assay. Finally, sugars were determined using reductimetric assay based on the reduction of Fehling solution by reducing sugars in hot acidic medium. These measurements were performed respectively according to the following norms: ISO2917, ISO 1442, ISO 1443, ISO937and Bertrand method.

III. Results and Discussion

Applied tests were done on a model food system to evaluate the effectiveness of bacteriocinogenic isolates [40]. It made necessary, the fact that, results provided by *in vitro* studies

does not necessarily guarantee the effectiveness of cultures for future food application [14], [41]. As regards, for simulations of real conditions of meat system, two tests could be used: (1) *ex situ*, by adding crude or semi-purified bacteriocin or (2) *in situ*, by adding bacteriocinogenic cultures [28]. In the current study, both tests were carried out.

The *ex situ* test as shown in Figs. 1 (a)-(b)-(c) confirmed the effectiveness of our antagonists metabolites as a very promising alternative for meat biopreservation. In the positive control batch, total viable counts showed values of $1.3x10^3$, $3.6x10^5$ and 10^7UFC/ml after 3, 6 and 9 days of incubation, respectively. The negative control batch showed no colony after 3 days of incubation, 10^5 and 10^7 UFC/ml after 6 and 9 days of incubation, respectively. Thus, the batches L_{m14}, L_{m24} and L_{m25} had a negative effect against total viable counts and also staphylococcus counts along the nine days of incubation.

On the L_{m14} batch total viable counts increased from $10^3$to $1.8x10^4$ and 10^7UFC/ml after 3, 6 and 9 days of incubation, respectively. Staphylococcal control batches were juxtaposed as from the sixth days of incubation. However, the enumeration varied from $1.3x10^4$ to $2.3x10^3$ UFC/ml and from $2.7x10^6$ to $1.8x10^6$UFC/ml for positive and negative control respectively. It should be noticed that the staphylococcal load had a regression of 0.73 log units at the sixth day of incubation comparing the positive batch to the one in contact withL_{m14}.

On the L_{m24}batch the total viable counts increased from 10^3 to $2.4x10^4$ and $4.2x10^7$ UFC/ml after 3, 6 and 9 days of incubation, respectively. Staphylococcus counts appeared distinct from that of positive control with a reduction of 2.11 log units at the sixth day of incubation.

As on L_{m25} batch total viable counts were $5x10^2$, $1.4x10^4$ and $2.9x10^7$UFC/ml after 3, 6 and 9 days of incubation, respectively. In this batch, staphylococcus counts showed the best result with a decrease of about 2.41 log units.

Quite similar results, after nine days of culture, were observed [28]. Nevertheless, future application of such isolates, in meat should not be done alone, but rather part of a system with other, hurdle technology, which has sure lead an increase in their activity [40].

The *in situ* test Figs. 2 (a)-(b)-(c)-(d) showed a relatively slow growth of lactic acid bacteria. This interesting result might made the tested isolates as goods candidates to extending shelf life of meat [42].

In addition, the tested isolates, displayed less inhibitory attitude when compared with *ex situ* test. Hence, the batches L_{m14}recordeda decrease in the total viable counts of $2.4x10^6$, 9.5x106 and $1.3x10^9$ UFC/ml. The lactic acid bacteria counts decreased too of$2.2x10^6$, $6.6x10^6$and $3.3x\ 10^7$ UFC/ml after 3, 6 and 9 days of incubation, respectively. However Staphylococcus counts moved from the positive control by a reduction of 0.73 log units.

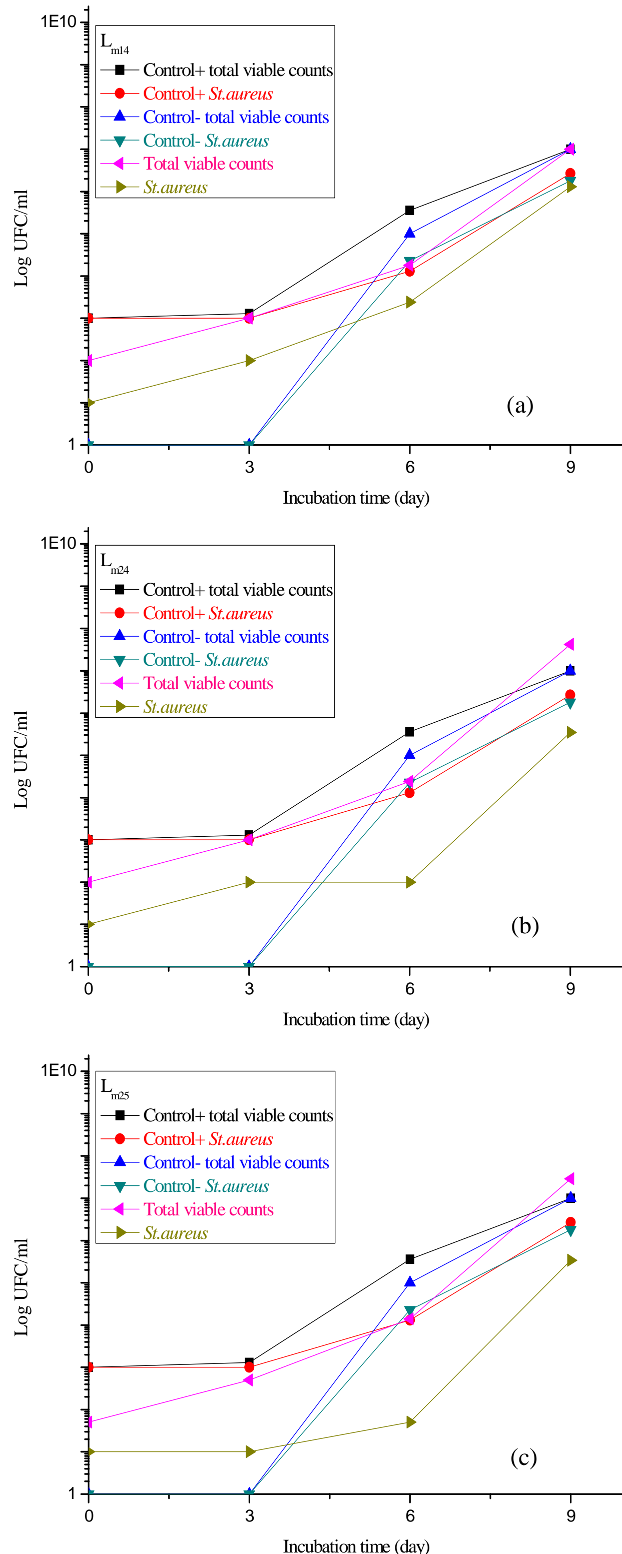


Fig. 1 Growth curves of the *ex situ* test for, (a) L_{m14}, (b) L_{m24} and (c) L_{m25}, at 7°C

Total viable counts in contact with L_{m24} showed an increase from $9.6x10^4$ to $2x10^6$ and $2.5x10^9$ UFC/ml. As for, lactic acid bacteria counts increased from $4.1x10^4$ to $2x10^4$ and $3x10^7$ UFC/ml after 3, 6 and 9 days of incubation, respectively. This batch showed a reduction of 0.20 log units at the sixth day of incubation.

On the L_{m25}batch, total viable counts increased from 2.10^5 to $4x10^5$ and $3.2x10^9$ UFC/ml. As for lactic acid bacteria increased from 10^5 to $5.4x10^5$ and $2.6x10^7$ UFC/ml after 3, 6 and 9 days of incubation, respectively. This batch showed a reduction of Staphylococcus counts of 0.86 log units, at the sixth day of incubation.

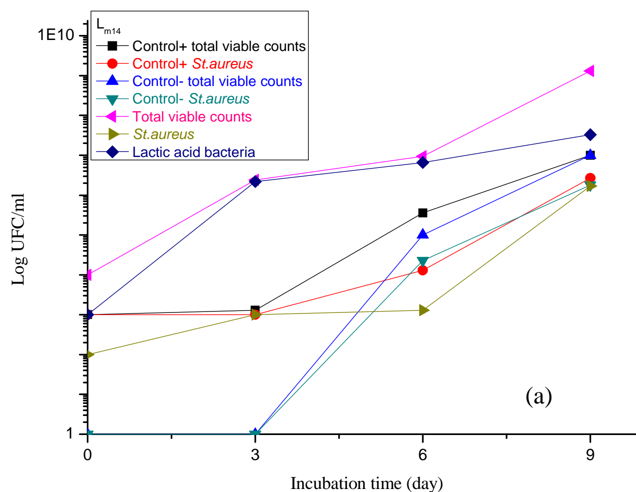
On the mix inoculation batch, total viable counts registered values of $2.1x10^5$, $1.4x10^5$ and $2.2x10^9$ UFC/ml. While, lactic acid bacteria countswere10^4, $2x10^4$ and $2.1x10^7$ UFC/ml after 3, 6 and 9 days of incubation, respectively. Staphylococcus counts showed a reduction of 0.032 log units at the sixth day of incubation compared with the positive control.

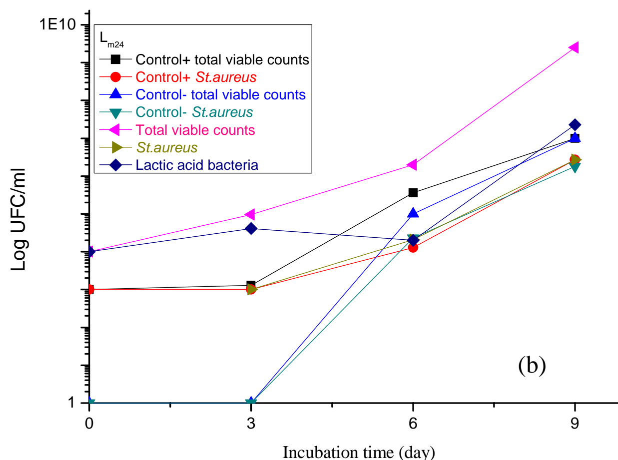
Smaller reduction was observed in the *in situ* test when compared with the *ex situ* one. Furthermore, total viable counts seemed more important in the presence of additional initial tested isolates.

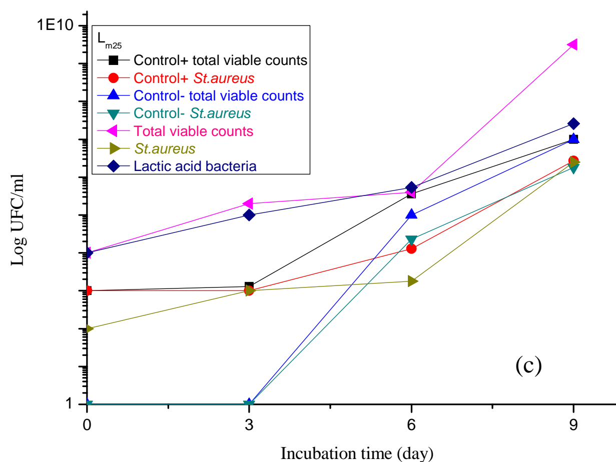
Physicochemical analyses could give more information in the presence of bio-protective cultures and have a negative influence on the sensory properties of meat. Thus, the pH increased from its normal value (5.50) to 5.77, 6.18, 5.96, and 7.22. However, dry mater decreased from 7.30% to 7.05%, 6.87%, 6.32 %, and 6.00 %. As well as fats and protein rated from 1.53% to 1.49%, 1.07%, 0.99%, 0.87%, and from 6.18% to 5.25, 5.56, 5.37, 5.5, for each one. These results were respectively obtained in the four batches L_{m14}, L_{m24}, L_{m25}, and $L_{m14+m24}$. The lower rate of dry mater was correlated to the decrease in fat and total protein. However, the increase of pH could be explained by the appearance of basic substances coming from degradation of the protein content of meat, particularly since sugars were present in small amounts 0.5%. These changes were certainly due to the metabolic activity of additional lactic acid bacteria.

IV. Conclusion

Our results indicated that the three bacteriocinogenic isolates: L_{m24}: *Lactobacillus sakei*, L_{m14}: *Pediococcus* spp., and L_{m25}produced an inhibitory compound “Bacteriocins”. When bacteriocins are used as crude solution they affect significantly the enterotoxin producer strain *St. aureus ATCC 6538*, and outcome in different percent reduction, from the beginning of the experiments till the ninth day of incubation, particularly at the sixth day with0.73, 2.11, and2.14 log units. When the isolates are used themselves they affect the pathogen in question too with minus decrease: 0.73, 0.20, 0.86, and 0.032log units. These constitute the two main important findings of the study since the safety and shelf life of meat are dictated by the time required for such as pathogenic bacteria to reach critical level.







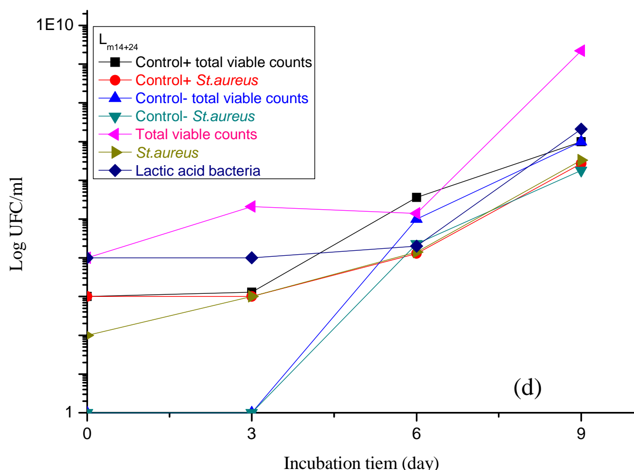


Fig. 2 Growth curves of the *in situ* test for, (a) L_{m14}, (b) L_{m24} and (c) L_{m25} and (d) $L_{m14+m24}$, at 7°C

The results obtained from the two tests suggest further experiments with higher and purified concentrations of bacteriocins to an efficient contribution to the hygienic-sanitary quality of meat and thus be an alternative to chemical preservatives.

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