Anti-Staphylococcal Enterotoxinogenesis of *Lactococcus lactis* in Algerian Raw Milk Cheese

Fatiha Tetili[1], Farida Bendali[1]*, Josette Perrier[2] and Djamila Sadoun[1]

[1]Applied Microbiology Laboratory, Life and Nature Sciences Faculty, Bejaia University, DZ-06000 Bejaia, Algeria

[2]Paul Cézanne Aix-Marseille III University, Group Molecular Interactions Intestinal Mucosal Microbiote, ISM2-Biosciences UMR CNRS 6263, Faculty of Sciences, FR-13397 Marseille Cedex 20, France

Summary

Staphylococcus aureus is a potential pathogen contaminating raw milk and dairy products, where it is able to produce thermostable enterotoxins that can cause staphylococcal food poisoning. This study was undertaken to investigate the inhibitory activity of a *Lactococcus lactis* strain (isolated from milk) on *S. aureus* growth and staphylococcal enterotoxin A (SEA) production. In the presence of *L. lactis*, the number of the pathogen decreased significantly (p<0.05) after 6 h of incubation in a laboratory medium and milk (3 log CFU/mL reduction compared to pure cultures). SEA concentration was reduced by 79 % in the co-cultures. *S. aureus* was unable to reach population levels permitting SEA production in the cheese inoculated with *L. lactis* during 32 days of storage. In contrast, during the same period, it attained 7 log CFU/g in the cheese manufactured without the lactococcal strain, a level which permitted SEA detection in the cheese extracts. However, this enterotoxin was never detected in the cheese harbouring *L. lactis*. These results demonstrate the anti-staphylococcal enterotoxinogenesis potential of the *L. lactis* strain and its usefulness in raw milk cheese biopreservation.

Key words: *Staphylococcus aureus* and *Lactococcus lactis* co-cultures, enterotoxin A, inhibitory activity, milk, cheese, storage

Introduction

Staphylococcus aureus is recognized as the major causative agent of food poisoning (*1*), which results from ingestion of foods containing one or more pre-formed toxins. Severe symptoms such as nausea, violent vomiting, diarrhoea and abdominal cramps may be developed by susceptible individuals following consumption of these toxins (*2*). Milk and dairy products have been incriminated in several outbreaks worldwide (*3*). Indeed, *S. aureus* is a common cause of udder diseases in dairy cows. In Algeria, it has been demonstrated that this bacterium is the major etiological agent of clinical and subclinical bovine mastitis (*4,5*). Presence of enterotoxigenic *S. aureus* strains in milk constitutes a high risk of food intoxication when it is consumed or used

for cheese making, especially if no thermal treatment is applied (*6*). Despite this hazard, some traditional fermented dairy products, including cheese, are still prepared in Algerian households using raw milk.

Besides mastitic milk (*7*), the processing plant environment and the workers may constitute serious *S. aureus* contamination sources during cheese making (*8,9*). Presence of *S. aureus* in milk has been largely reduced by identifying and controlling the potential sources of this species, especially mastitis and lack of hygiene (*10*). However, *S. aureus*, even when present at low levels in milk, can reach over 5 log CFU/g during the early stages of cheese making (*11*). It was reported that 25 % of *S. aureus* strains, isolated from food, produced heat-stable enterotoxins (*12,13*). These toxins are a family of exoproteins forming a

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*Corresponding author: Phone/Fax: +213 (0)34 813 710; E-mail: kamelea03@hotmail.com

ORCID IDs : 0000-0003-4116-4696 (Tetili), 0000-0002-1891-8421 (Bendali), 0000-0003-1894-8492 (Perrier), 0000-0002-6153-1660 (Sadoun)

single chain with low molecular mass, ranging from 26 to 30 kDa (*14*). Twenty-four different staphylococcal enterotoxins (SEs) and SE-like (SEls) toxins have been reported (*15*), and most of the genes coding for them have been described (*16,17*). Contrary to the producer microorganism, these enterotoxins are remarkably heat-resistant; they resist the temperatures which easily destroy the bacteria that produce them as well as the proteolytic enzymes, and hence retain their activity in the digestive tract after ingestion (*18,19*).

Staphylococcal enterotoxin A (SEA) is responsible for 75 % of staphylococcal food poisoning outbreaks in developed countries (*20*), followed by staphylococcal enterotoxin D (SED, 37.5 %) and staphylococcal enterotoxin B (SEB, 10 %). It was reported that a dose of about 40 ng of SEA or SED is sufficient to induce intoxication (*3*). Therefore, to ensure the safety of raw milk cheese, growth of *S. aureus* must be controlled. In 2006, the European legislation laid down the obligation to determine the presence of enterotoxins in cheese when the level of *S. aureus* is over 5 log CFU/g (*21*). Thus, it is very important to investigate bacterial interactions to understand the mechanisms by which the inhibition of pathogens by other bacterial species may occur. It is well documented that some lactic acid bacteria (LAB) are able to inhibit *S. aureus* growth (*22,23*) mainly by production of organic acids (lactic acid). Both decreasing pH (acidification) and increasing undissociated lactic acid concentration have been reported to exert the inhibitory effects on pathogens. To the best of our knowledge, there are few reports on the inhibition of SEA production especially by LAB. The manufacturers of raw milk cheese rely on the biopreservative action of the sole autochthonous flora of the used milk or on the addition of selected strains with antimicrobial potential. Therefore, the main aims of this study are the manufacture of fresh raw milk cheese by applying a selected anti-staphylococcal *Lactococcus lactis* strain and the evaluation of its inhibitory effect on *Staphylococcus aureus* growth and SEA production.

Materials and Methods

Lactococcus lactis identification and growth conditions

A strain of *L. lactis*, originally isolated from raw milk, collected from a healthy cow in Bejaia city (Algeria), and selected on the basis of its strongest anti-*Staphylococcus* activity, was used in this study. It was cultivated in M17 broth and agar (Difco, Le Pont de Claix, France) at 30 °C. This strain was identified by 16S rDNA sequencing. Briefly, genomic DNA was extracted using Qiagen kit (Qiagen, Hilden, Germany) as follows. A volume of 2 mL of an overnight culture of the strain was centrifuged at 5000×*g* (Sigma 1–14; Sigma Zentrifugen, Osterode am Harz, Germany) for 10 min and the supernatant was discarded. The pellet was then suspended in 180 µL of enzymatic lysis buffer (Qiagen). The extraction was continued following the manufacturer's instructions. 16S rRNA gene was amplified by polymerase chain reaction (*24*) using universal primers (Qiagen) (Table 1).

Table 1. Oligonucleotide primers used for *Staphylococcus aureus* and *Lactococcus lactis* identification

Strain	Primer	Sequence
S. aureus	OlF515	5' GTGCCAGC(AC) GCCGCGG 3'
	OlF915	5' A(GT) GAATTGACGGGG(AG) C 3'
L. lactis	OlR1406	5' ACGGGCGGTGTGT(AG) C 3'
	OlR930	5' G(CT) CCCCGTCAATTC(AC) T 3'

Staphylococcus aureus isolation, identification and growth conditions

Raw milk samples for *S. aureus* isolation were collected from cows with subclinical mastitis in different farms located in Bejaia city (Algeria). Milk samples were collected aseptically into sterile 250-mL bottles after disinfection of the mammary udders. Before isolation, enrichment for staphylococcal growth was carried out in Giolitti--Cantoni broth (GC; Pasteur Institute of Algeria, Algiers, Algeria) supplemented with 1 % (by volume) potassium tellurite (Pasteur Institute of Algeria) and incubated at 37 °C for 24 h under anaerobic conditions. Isolation was carried out on mannitol salt agar (Bacto™, Le Pont de Claix, France) and plates were incubated at 37 °C for 24--48 h under aerobic conditions. Characteristic colonies were identified phenotypically as previously described (*25*) using Gram staining, catalase, coagulase and DNase tests. The selected strains were identified by 16S rRNA gene sequencing. Genomic DNA was extracted using Qiagen kit. 16S rRNA gene was amplified by polymerase chain reaction (*24*) using universal primers (Qiagen) (Table 1). Strains were stored on nutrient agar (NA; Sigma-Aldrich Chemie, Steinheim, Germany) at 4 °C until use. For long--term storage, the strains were stored in brain heart infusion broth (BHI; Sigma-Aldrich Chemie) supplemented with 30 % (by volume) glycerol (Sigma-Aldrich Chemie) at -20 °C.

In vitro anti-staphylococcal activity of L. lactis

Agar diffusion methods

L. lactis antibacterial activity against *S. aureus* was detected using the spot-on-lawn and the well diffusion methods (*26*). Briefly, *L. lactis* was cultured overnight in M17 broth (Difco) at 30 °C. An aliquot (5 µL) of *L. lactis* culture was spotted onto M17 agar and incubated at 30 °C for 18 h. Then the plates were overlaid with 10 mL of Müller-Hinton agar (MH; Pasteur Institute of Algeria) inoculated with the *S. aureus* strain at a final concentration of 5 log CFU/mL and re-incubated at 37 °C for 24 h. Finally, appearance of the inhibition zone around the spots was observed. For well diffusion method, *L. lactis* cell-free supernatant (CFS) was recovered from an overnight culture (9 log CFU/mL) centrifuged at 8000×*g* for 20 min at 4 °C (Sorvall RC-5C Plus, Thermo Fisher Scientific, Bad Homburg, Germany) and then filter-sterilized through 0.22-µm Millipore filters (Merck Millipore, St. Quentin en-Yvelines Cedex, France). MH agar plates were inoculated with *S. aureus* (5 log CFU/mL) and cooled at 4 °C for 30 min, then wells (6 mm in diameter) were made and filled with *L. lactis* CFS (100 µL). To allow

diffusion of the tested supernatant, the plates were pre-incubated at 4 °C for 2 h. At the end of the incubation period (37 °C for 18 h), the anti-staphylococcal activity was estimated by measuring the inhibition zone diameters. Neutralized CFS (pH=6.5, 3 mol/L of NaOH) was also tested using the same method.

Co-cultures in M17 broth and milk

In this test, the study of the *in vitro* antagonism of the lactococcal strain against *S. aureus* was performed by co-culture in M17 broth or skimmed milk to assess the inhibitory potential of *L. lactis* strain when it coexists with *S. aureus*. The latter was cultivated in 9 mL of M17 broth or skimmed milk at 37 °C overnight. After incubation, it was inoculated into 100 mL of the same medium to have a final count of 6 log CFU/mL (the highest level of *S. aureus* enumerated in the mastitic raw milk samples used for isolation of the *S. aureus* strain) in 250-mL glass flasks. Like *S. aureus*, *L. lactis* was pre-cultured in 9 mL of M17 broth or skimmed milk at 30 °C overnight, and then inoculated into 100 mL of the same medium (final level of 8 log CFU/mL, the mean count of lactic acid bacteria, including lactococci, enumerated in our raw milk samples), previously inoculated with *S. aureus* as described above. Similarly, pure cultures of *L. lactis* and *S. aureus* were prepared in the same media and at the same final cell levels. Pure and co-cultures were incubated at 30 °C under aerobic static conditions. Culture aliquots (10 mL) were periodically sampled for *S. aureus* viable cell enumeration on mannitol salt agar (Bacto™), pH determination (universal pH indicator paper, pH=1-14; Merck KGaA, Gernsheim, Germany) and SEA detection (in M17 broth). Similarly, growth of *L. lactis* in pure cultures was followed at the same time intervals. The measurements were carried out at 0, 2, 4, 6, 8, 10, 12 and 24 h of incubation in three independent experiments.

Enzyme-linked immunosorbent assay (ELISA) test for SEA detection and quantification

Staphylococcal enterotoxin A (SEA) was detected in the CFS obtained from *S. aureus* pure and co-cultures in M17. CFS was recovered as indicated above and stored at 4 °C until analyses. SEA was first detected in CFS obtained from a 24-hour culture, then quantified in CFS obtained after each incubation period (2, 4, 6, 8, 10 and 12 h) from *S. aureus* pure and co-cultures (with *L. lactis*) using ELISA test. This test was performed in 96-well polystyrene plates (Greiner Bio One, Frickenhausen, Germany). The protocol was as follows: first, the polystyrene plate wells were coated overnight at 4 °C with 100 µL of CFS and pure SEA (Sigma-Aldrich, St. Louis, USA) at concentrations from 5 to 100 ng/mL (with an increment factor of 5) as positive controls. Second, the plates were saturated with 150 µL of phosphate buffered saline-bovine serum albumin (PBS-BSA) 1 % (by mass per volume) for 1 h at room temperature. Third, 100 µL of diluted (1:500 in 1 % (by mass per volume) PBS-BSA) rabbit anti-SEA antibody (Sigma-Aldrich) were added to each well. The plates were incubated at 37 °C for 2 h and then washed three times with PBS containing 0.1 % (by volume) Tween 20 (Sigma-Aldrich). Forth, the secondary antibody: 100 µL of diluted (1:1000) anti-rabbit immunoglobulin G (whole molecule) alkaline-phosphatase-conjugate antibody developed in goat's serum (Sigma-Aldrich) were added to each well and the plates were left for 1 h at room temperature. Finally, for colorimetric detection, 100 µL of the phosphatase substrate, *p*-nitrophenylphosphate at 1 mg/mL in diethanolamine buffer pH=9.6 (Sigma-Aldrich), were added per well. The absorbance was read at 405 nm with an automatic ELISA plate reader (Power WaveX 340; Bio-Tek Instruments, Inc., Winooski, VT, USA) after 30 min at room temperature. In the same step, to quantify the amount of SEA in CFS and obtain SEA standard curve, pure SEA solutions at increasing concentrations (0.75-100 ng/mL) were incubated separately. The amount of SEA (ng/mL) in CFS was determined from the standard curve.

In situ anti-staphylococcal activity of L. lactis

Cheese manufacturing

Cheese samples were made from raw cow's milk. Before cheese making, the presence of *S. aureus* in raw milk was assessed. Milk at 30 °C (pH=6.5) was divided equally in three 2-litre vats; the first one was inoculated with *S. aureus* solely (3 log CFU/mL, the mean level of *S. aureus* enumerated from naturally contaminated raw milk), whereas the second vat was inoculated simultaneously with *S. aureus* (3 log CFU/mL) and *L. lactis* (8 log CFU/mL). A non-inoculated vat was included as a control to assess any *S. aureus* contamination during the cheese making. Rennet (Caglificio Clerici Spa., Cadorago, Italy) was added to milk (1.5 µg/L) one hour after the culture inoculation. Forty minutes after rennet addition, the curd was cut and whey was drained off. The obtained curd was distributed into cylindrical perforated stainless steel moulds and left for 6 h at room temperature, then immersed in 20 % (by mass per volume) brine for 30 min, and maintained for 2 h at room temperature. Finally, the cheese samples were stored at 8 °C for 32 days.

Staphylococcus aureus count in cheese

Viable cells of *S. aureus* in cheese were counted every day during the first week of storage and then on days 14, 21, 28 and 32. Cheese samples (5 g) were homogenized in 45 mL of sterile saline water (9 g/L NaCl) at 45 °C and then serial decimal dilutions were carried out and plated in triplicate on mannitol salt agar (Bacto™). After incubation (at 37 °C for 48 h), the *S. aureus* number was determined and expressed in CFU per g of cheese.

SEA extraction and detection in cheese

Staphylococcal enterotoxin A was extracted following the procedure of Soejima *et al.* (*27*) and detected using Western blot test with some modifications. Briefly, 20 g of cheese were combined with 40 mL of distilled water (37 °C) and homogenized. The suspension was adjusted to pH=3.8-4 with 2 mol/L of HCl (Sigma-Aldrich) and left for 10 min at room temperature, then centrifuged at 3000×*g* (Sigma 1–14; Sigma Zentrifugen) for 20 min at 4 °C. The supernatant was adjusted to pH=6.6-6.8 with 2 mol/L of NaOH (Sigma-Aldrich). Chloroform (Sigma-Aldrich) was added to the supernatant at a

final volume fraction of 10 %; the mixture was vigorously shaken for 20 s and then centrifuged at 3000×*g* (Sigma 1–14; Sigma Zentrifugen) for 20 min at 4 °C. Cold trichloroacetic acid (Sigma-Aldrich) was added at a final mass per volume ratio of 20 % to the obtained supernatant. After cooling on ice for 30 min, the mixture was centrifuged again (3000×*g* for 30 min at 4 °C), the pellet was resuspended in Tris (Sigma-Aldrich) buffer (0.3 M, pH=8) and then stored at -20 °C until analyses. Western immunoblot analysis was done with the polyclonal antiserum prepared against SEA. Samples were electrophoresed through a 12 % (by mass per volume) sodium dodecyl sulfate polyacrylamide gel with a 6 % (by mass per volume) stacking gel (*28*) and electrophoretically transferred to nitrocellulose membrane (Whatman® Protran®; Sigma-Aldrich, Dassel, Germany). Cheese extracts were boiled for 10 min just before being loaded onto the 12 % SDS-PAGE gel. Protein molecular mass standards (Euromedex, Souffelweyersheim, France) were also loaded onto the gel. The Bio-Rad mini gel (1.5) apparatus (Bio-Rad, Marnes-la-Coquette, France), used for SDS-PAGE, was run for 1 h at 180 V, and then the gels were transferred to the nitrocellulose membrane at 100 V for 1 h. The membrane was blocked with 5 % Tris-buffered saline-bovine serum albumin (TBS-BSA; Thermo Scientific™, Thermo Fisher Scientific, Inc., Waltham, NA, USA) for 1 h, incubated with rabbit anti-SEA antibody (Sigma-Aldrich) and raised against native SEA, diluted at 1:500 (by volume) in TBS-BSA for 1 h. After washing, the membrane was incubated with an anti-rabbit horseradish peroxidase (HRP; Jackson ImmunoResearch, West Grove, PA, USA) diluted at 1:1000 (by volume) in TBS-BSA for 1 h. Following a second washing, the mixture of HRP substrate SuperSignal West Pico Stable Peroxide/SuperSignal West Pico Luminol/Enhancer solution (Thermo Scientific™, Thermo Fisher Scientific, Inc.) was added and the Western blots were scanned using a Fluor Chem Q (Cell Biosciences, Santa Clara, CA, USA) apparatus.

Statistical analysis

All the experiments were performed in triplicate and results were expressed as mean value±standard deviation. Statistical analysis was performed using one-way analysis of variance (ANOVA) together with the Tukey's test using the STATISTICA v. 10 (StatSoft, Paris, France) software. Values of $p < 0.05$ were considered significant.

Results and Discussion

Lactococcus lactis exerts a significant anti-staphylococcal activity in vitro

Lactococcus lactis was found to possess an antibacterial activity against *Staphylococcus aureus* following the spot-on-lawn and the well diffusion tests with inhibition zone diameters of 17 (Fig. 1) and 10 mm (not shown), respectively. However, these inhibition zones disappeared when testing the neutralized cell-free supernatant (CFS), which indicates the effect of acidic pH on the antibacterial activity.

Fig. 1. Anti-staphylococcal activity of *Lactococcus lactis* strain (inhibition zone diameter of 17 mm) following the spot-on-lawn test

This activity was also demonstrated in M17 broth (Fig. 2) and milk co-cultures (Fig. 3). *S. aureus* growth in M17 broth (pure cultures) was biphasic; it was exponential until 12 h of incubation with a population reaching approx. 9.1 log CFU/mL, then stationary with stable cell number during the 24-hour culture period (approx. 9.1 log CFU/mL; Fig. 2a). In the co-cultures with *L. lactis*, *S. aureus* growth was similar to that observed in the pure cultures during the first four hours of incubation, with a population level of 7.4 log CFU/mL in both cultures. However, after 6 h of incubation, a significant decrease ($p < 0.05$) in the *S. aureus* viable cell number was registered and it reached 6 log CFU/mL at the end of the growth period (24 h), with 3-log reduction compared to the pure cultures (Fig. 2a).
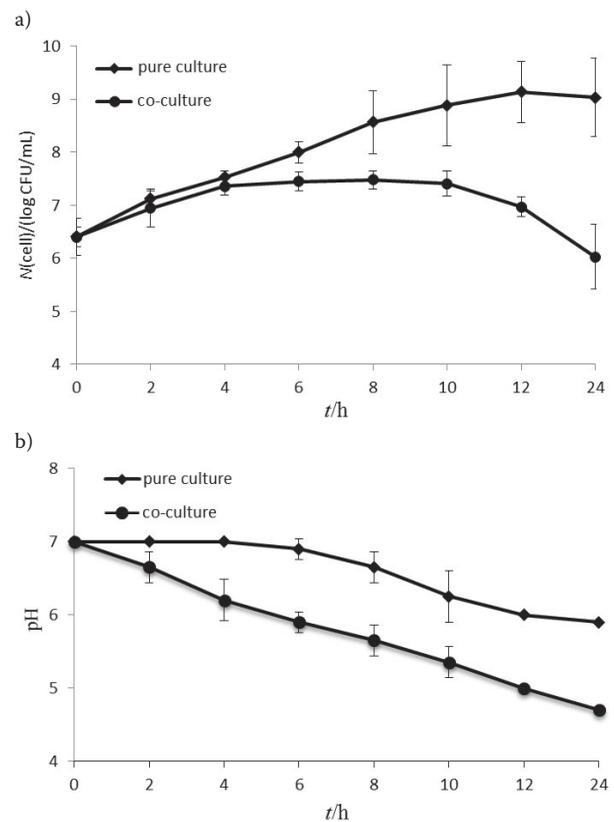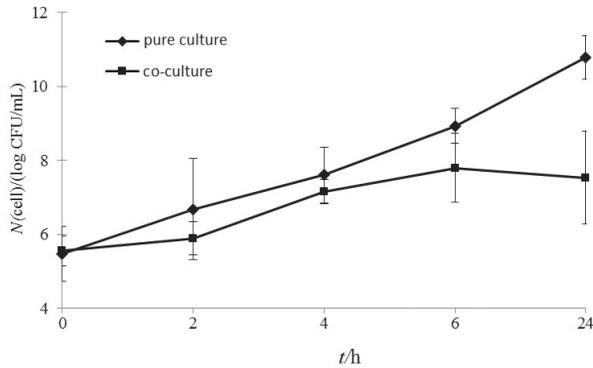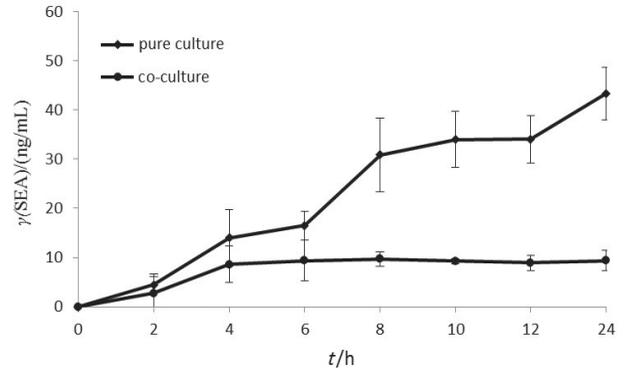

Fig. 2. Growth kinetics of *Staphylococcus aureus* (a) and pH evolution (b) in pure and co-cultures in M17 broth. Values are the average of three independent experiments. Error bars correspond to standard deviations

Fig. 3. Growth kinetics of *Staphylococcus aureus* in pure and co-cultures in skimmed milk at 30 °C. Values are the average of three independent experiments. Error bars correspond to standard deviations


Fig. 4. Staphylococcal enterotoxin A (SEA) production at 37 °C in pure and co-cultures in M17 broth as detected by ELISA test in the supernatants. Values are the average of three independent experiments. Error bars correspond to standard deviations

This inhibitory effect was concomitant to a significant pH drop in the co-cultures. Indeed, pH evolution during pure and co-cultures in M17 broth was determined every 2 h (Fig. 2b). The pH value decreased in the *S. aureus* pure cultures by only one unit (from pH=7 to pH=6) after 24 h of incubation. On the contrary, in the co-cultures with *L. lactis* (8 log CFU/mL) the pH was much lower (pH=4.9) at the end of the culture period (24 h; Fig. 2b). This decrease in the pH value in the co-cultures may be explained by the glucose metabolisms of both strains. According to the classification established by Charlier *et al.* (*29*) based on *S. aureus* population and pH value reductions, our *L. lactis* strain could be classified in group II (with low acidification and strong inhibition potential). Therefore, *L. lactis* seemed to exert a good inhibitory effect on the *S. aureus* growth; acidification and accumulation of undissociated lactic acid form may be the main plausible mechanisms involved in this effect. Similarly, when cultured in skimmed milk with *L. lactis* (Fig. 3), *S. aureus* viable cells reached a count of 7.14 log CFU/mL after 4 h of incubation. This count remained stable throughout the culture, contrary to the pure cultures where *S. aureus* average level was 10.78 log CFU/mL after 24 h of incubation. This difference may be due to the inhibitory activity of *L. lactis* as observed in M17 broth.

The antibacterial activity of *L. lactis* is well documented in the literature and many strains have been recognized for their anti-staphylococcal action (*29–32*). Similar results were reported by Even *et al.* (*33*) and Samelis *et al.* (*34*), and inhibition of *S. aureus* growth in milk was already reported by Muñoz *et al.* (*6*), Charlier *et al.* (*35*) and Le Marc *et al.* (*36*). Some studies have shown that co-culture with lactic acid bacteria reduced *S. aureus* growth following nutritional competition. Indeed, these bacteria (including *L. lactis*) are able to metabolize different carbon sources, such as glucose and lactose, which are also used by *S. aureus* (*29*). Furthermore, competition for vital nutritive elements, such as biotin and niacin, was shown to be responsible for the *S. aureus* inhibition in co-cultures with *L. lactis* (*29*). This anti-staphylococcal effect may also be due to specific substances produced by *L. lactis*, mainly organic acids, leading to medium acidification (*35,36*).

Inhibition of SEA production by *Lactococcus lactis*

Parallel with the growth monitoring, SEA production was followed in M17 broth pure and co-cultures, and it was quantified using ELISA test. SEA was detected in the CFS after 2 h of incubation (Fig. 4). Derzelle *et al.* (*37*) have reported that SEA can be produced during all *S. aureus* growth phases. Similarly, Rasooly and Rasooly (*38*) demonstrated that SEA was produced after 160 min of culture in BHI broth. In pure *S. aureus* cultures, SEA production increased progressively and reached 30.83 and 34.05 ng/mL after 8 and 12 h of incubation, respectively. After 24 h of incubation, toxin concentration was about 43.5 ng/mL. However, in the co-cultures with *L. lactis*, SEA level was significantly (p<0.05) lower than that determined in the pure cultures. It was lower than 10 ng/mL throughout the incubation period (24 h). In these cultures, SEA level was reduced by 79 % (<10 ng/mL), which demonstrates that toxin production was inhibited by *L. lactis*. Even *et al.* (*33*) have reported that changes in the expression of enterotoxins A and L genes (*sea* and *sel*) occurred in co-cultures of *S. aureus* with *L. lactis* ssp. *lactis* biovar. *diacetylactis* LD61 in a chemically defined medium. Furthermore, Rosengren *et al.* (*39*) have demonstrated that accumulation of undissociated lactic acid exerts a significant inhibitory effect on SEA production. This could explain our results since the pH decreased to pH=4.9 in the presence of *L. lactis* strain (Fig. 2b).
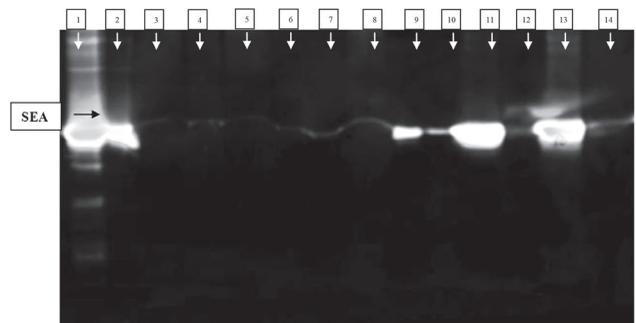
Inhibition of *S. aureus* growth and SEA production in cheese

To investigate the anti-staphylococcal activity of the lactococcal strain *in situ*, the latter was applied to manufacture a fresh cheese using raw milk experimentally contaminated with *S. aureus*. For this, raw milk was firstly analyzed for *S. aureus* presence and no cell was detected. The milk was then inoculated with 3 log CFU/mL of *S. aureus* and its growth was monitored. A maximum of 5.39 log CFU of *S. aureus* per g of cheese was reached after one day of cheese manufacture, but this level was maintained at the initial level (3 log CFU/mL) in the cheese made with the addition of *L. lactis* (Fig. 5). This different behaviour was observed throughout the first week after inoculation;
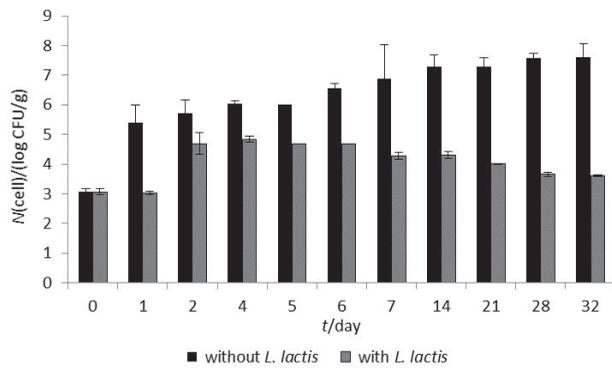
Fig. 5. *Staphylococcus aureus* strain counts in cheese manufactured without and with *Lactococcus lactis* strain and stored at 6 °C for 32 days. Values are the average of two independent experiments. Error bars correspond to standard deviations

Fig. 6. Staphylococcal enterotoxin A (SEA) detection in cheese extracts by the Western blot analysis following storage at 8 °C for 32 days. lane 1=molecular mass ladder, lane 2=γ(SEA$_{pure}$)=50 ng/µL, lanes 3, 5, 7, 9, 11 and 13=extracts from cheese made with only *S. aureus* at 0, 7, 14, 21, 28 and 32 days of storage, respectively, lanes 4, 6, 8, 10, 12 and 14=extracts from cheese made with *S. aureus* and *L. lactis* at 0, 7, 14, 21, 28 and 32 days of storage, respectively

indeed, *S. aureus* counts increased progressively in the two types of cheese but obtained 6.87 and 4.3 log CFU/g respectively at the end of this storage period. Similarly, on the 32nd day of storage, *S. aureus* cell number in cheese made with *L. lactis* (3.6 log CFU/g) was significantly ($p<0.05$) lower (4 log units) than that recorded in cheese containing only the pathogen bacterium (7.6 log CFU/g). Therefore, cheese manufactured with *L. lactis* was significantly ($p<0.05$) less populated with *S. aureus* than the cheese made without this lactic strain. According to these results, the lactic strain used in this study was highly effective in controlling *S. aureus* growth in cheese. This effectiveness was well highlighted by the significant decrease of the viable *S. aureus* counts from the beginning until the end of the storage period (32 days). As mentioned in Materials and Methods, a control cheese made with non-inoculated raw milk was included in this trial and non-*S. aureus* cells were detected during the manufacture and throughout the storage period. Parallel with *S. aureus* cell count monitoring, SEA production was inspected in cheese extracts using the Western blot system. It was detected after 21 days of storage in the extracts of cheese made without *L. lactis* and remained detectable until the end of storage (Fig. 6). This observation is in agreement with the staphylococcal count evolution in the cheese. During this period, the number of viable staphylococcal cells was around 7 log CFU/g. According to Fujikawa and Morozumi (*40*), SEA could be detected when *S. aureus* population was over 6 log CFU/mL. Conversely, extracts from cheese made with *L. lactis* were devoid of SEA. It was never detected during storage period since *S. aureus* was below the permitted SEA detection level (<6 log CFU/g). This result may be related to the antibacterial activity of the lactic strain against *S. aureus* growth and SEA production, also exerted in cheese. It was reported that in artisanal cheese, *L. lactis* was a predominant flora with a good acidification and an ability to produce antimicrobial compounds (*41*). However, in the study of Hamama *et al.* (*42*), when *S. aureus* was present at high levels (>5 log CFU/mL) in the milk, staphylococcal enterotoxins were rapidly formed in the cheese, even in the presence of a nisin-producing lactic starter. Consequently, raw milk quality and good conditions of manufacture are primordial to insure cheese safety. This study emphasized

the importance of maintaining the *S. aureus* counts in milk at less than 3 log CFU/mL. The active growth of *S. aureus* in cheese (in the absence of *L. lactis*) during storage indicates the possibility of a public health hazard, despite the low contamination level of milk, and so implicates the presence of staphylococcal enterotoxins in raw milk cheese. On the contrary, in cheese made with *L. lactis*, *S. aureus* growth was drastically inhibited, and this pathogen did not attain a population level that would allow SEA production. It was at a level lower than 5 log CFU/g during the entire storage period.

Conclusions

Lactococci (*Lactococcus lactis*) are largely used as industrial or artisanal starter cultures for the manufacture of many cheese types, including fresh and soft cheese. To our knowledge, this is the first report where *L. lactis*, the model LAB, isolated from Algerian cow's milk, has been shown to have a drastic inhibitory effect on *S. aureus* growth and staphylococcal enterotoxin A (SEA) production in cheese. This inhibition was demonstrated in a laboratory medium and in milk where *S. aureus* growth was significantly reduced in co-culture with *L. lactis* strain. Consequently, SEA production was also decreased. The lactic strain studied in this work was found to effectively inhibit *S. aureus* growth and SEA production during raw milk cheese storage (32 days). As shown in this study, *L. lactis* strain is capable of preventing *S. aureus* growth and subsequent enterotoxin A production in cheese, particularly when the initial milk contamination with *S. aureus* is lower than 3 log CFU/mL. This finding confirmed the great importance of the use of this lactic strain in cheese making and preservation.

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