

Research Paper

Presence of enterotoxigenic *Staphylococcus aureus* in artisan fruit salads in the city of San Luis, Argentina

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Abstract

An increase in the consumption of fruit juices and minimally processed fruits salads has been observed in recent years all over the world. In this work, the microbiological quality of artisan fruit salads was analysed. Faecal coliforms, *Salmonella* spp, *Shigella* spp, *Yersinia enterocolitica* and *Escherichia coli* O157:H7 were not detected; nevertheless, eleven strains of *Staphylococcus aureus* were isolated. By multiplex PCR, all isolates showed positive results for *S. aureus* 16S rRNA gene and 63.6% of them were positive for *sea* gene. Furthermore, PCR *sea* positive strains were able to produce the corresponding enterotoxin. Finally, the inactivation of these strains in fruit salads by nisin, lysozyme and EDTA, was studied. EDTA produced a total *S. aureus* growth inhibition after 60 h of incubation at a concentration of 250 mg/L. The presence of *S. aureus* might indicate inadequate hygiene conditions during salad elaboration; however, the enterotoxigenicity of the strains isolated in this study, highlights the risk of consumers' intoxication. EDTA could be used to inhibit the growth of *S. aureus* in artisan fruit salads and extend the shelf life of these products.

Key words: *Staphylococcus aureus*, enterotoxigenic strains, fruit salads, antimicrobial compounds, multiplex PCR.

Introduction

An increase in the consumption of fruit juices and minimally processed fruits, in the form of peeled, cut and packaged fruit salads, has been observed in recent years. Fruit salads are not heat-processed, contain no preservatives, and therefore, they can be easily spoiled by fungi and bacteria. Many microorganisms, in particular acid-loving or acid-tolerant bacteria and fungi (yeasts and moulds), can use fruits as substrate and cause spoilage, producing flavour and odour alteration, product discoloration, and human illness if the contaminating microorganisms are pathogens (Tournas *et al.*, 2006). Possible contamination sources are soil, faeces, manure, irrigation and washing water, ice, animals (including insects and birds), handling of the harvesting and pro-

cessing equipment, and transport (Johnnessen *et al.*, 2002). Raw fruits and vegetables have been identified as carriers of pathogenic bacteria such as *Shigella* spp, *Salmonella* spp, enterotoxigenic and enterohemorrhagic *Escherichia coli*, *Staphylococcus aureus*, *Campylobacter* spp, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Bacillus cereus*, and parasites as *Giardia lamblia*, *Cyclospora cayetanensis* and *Cryptosporidium parvum* (De Roever *et al.*, 1998; Kumar *et al.*, 2006).

S. aureus is one of the major pathogens that can cause food poisoning. Intoxication is caused by the ingestion of enterotoxins within foods, usually because the food has been left at room temperature (Walls and Scott, 1997). There are five major classical types of staphylococcal enterotoxins (SEs): SEA, SEB, SEC, SED and SEE, as well

as new SEs or SE-like superantigens (Sags) such as SEG to SEU (Chiang *et al.*, 2008). Foods requiring considerable handling during preparation and kept without refrigeration are usually involved in staphylococcal poisoning. This bacterium is able to grow in a wide temperature range (7–48 °C), with an optimal growth at 35–37 °C, a frequent value in warm climates (Baeza *et al.*, 2007).

The increasing demand for high-quality, non-thermally processed and microbiologically safe foods requires the development of different treatments for the reduction of microorganisms. Nisin is a bacteriocin that forms pores in cell membranes, it has “generally recognized as safe” (GRAS) status, and has been approved for use as a food preservative (Montville and Chen, 1998). Lysozyme is a protein with hydrolytic activity (Mckenzie and White, 1991), although it has lately been reported to have an anti-bacterial activity that is independent of its enzymatic activity (Ibrain *et al.*, 2001). Sodium ethylenediamine tetraacetate (EDTA) is a chelating agent used in a wide variety of foods to prevent oxidation and other deteriorating reactions catalyzed by metallic ions. It has been claimed that the antimicrobial spectrum and potency of these molecules can be increased when used in combination each another (Chung and Hancock, 2000; Branen and Davidson, 2004) or other antimicrobials (Carneiro De Melo *et al.*, 1998; Gill and Holley, 2003).

The aims of this work were i) to study the microbiological quality of artisan fruit salads manufactured in our region, ii) to detect the presence of *sea*, *seb*, *sec*, *sed*, *see* genes encoding staphylococcal enterotoxins by multiplex PCR in our *S. aureus* isolates, and iii) to assess the inhibitory capacity of nisin, lysozyme and EDTA on this bacterium.

Materials and Methods

Sample collection

A total of 71 samples of artisan fruit salads obtained in retail shops in San Luis city, Argentina, were processed. The samples were stored at 4 °C for no longer than 4 h until processing. The pH of the samples was determined with an Orion model 420A pHmeter (Orion Research Inc., Boston, Massachusetts, USA).

Microbiological analysis

Twenty-five grams of each sample were homogenized in a stomacher (IUL Masticator, Koningswinter, Germany) for 1 min. From each homogenate, two serial decimal dilutions, 10^{-1} and 10^{-2} , were prepared in 0.1% peptone water pH 7 (PW, Britania Laboratories, Buenos Aires, Argentina).

Counts of total mesophilic aerobes

Volumes of 0.1 mL of homogenate and each dilution were spread in duplicate onto plate count agar (PCA, Merck

Laboratories, Darmstadt, Germany) and incubated at 37 °C for 24 h.

Counts of moulds and yeasts

Aliquots of 0.1 mL of homogenate and dilutions were surface spread onto duplicated oxytetracycline-glucose-yeast extract agar (OGY, Merck) and incubated at 22 °C for 5 days.

Counts of total and faecal coliforms

Total (TC) and faecal (FC) coliforms were investigated in Mac Conkey broth (Merck) at 37 °C for 48 h by the three-tube Most Probable Number (MPN) method. Presumptive results of TC were confirmed in bile brilliant green lactose broth (BGLB, Merck) at 37 °C for 24 h. FC were confirmed in *Escherichia coli* (EC) broth (Merck) at 44.5 °C for 24 h (De la Canal, 2004), with subsequent isolation on Eosin Methylene Blue agar (EMB; Merck). Suspect colonies from EMB were studied by Gram-staining and biochemical tests.

Investigation of *Salmonella* spp and *Shigella* spp.

These bacteria were investigated as described: 1 mL of homogenate were seeded into 9 mL of lactose broth (Merck) and incubated for 24 h at 37 °C. Then, one millilitre aliquots were transferred to two tubes containing 9 mL of selenite broth (Merck), and two tubes with 9 mL of tetrathionate broth (Merck). One tube of each selective broth was incubated 24 h at 37 °C and the other one was incubated 24 h at 42 °C. Isolations were done on Bismuth Sulphite agar (BiSA, Merck) and Salmonella Shigella agar (SS, Merck). Suspect colonies were assayed by Gram staining and classical biochemical tests (Caffer and Terragno, 2001; Tiruneh, 2009).

Counts of *E. coli* O157:H7

Volumes of 0.1 mL of homogenate and dilutions were seeded on Mac Conkey Sorbitol agar (SMC, Merck) at 37 °C for 24 h and sorbitol non-fermenting colonies were studied by biochemical tests and challenged against O157 antiserum.

Investigation of *Y. enterocolitica*

One millilitre of homogenate was inoculated in phosphate buffered saline (PBS, Merck) pH 7.6 added with 1% sorbitol and 0.15% bile salts for 21 days at 4 °C. Isolations were performed on Mac Conkey agar (MC, Merck) for 48 h at 22 °C. Presumptive colonies were subjected to Gram staining and classical biochemical tests according to Bercovier and Mollaret (1984).

Counts of *S. aureus*

Aliquots of 0.1 mL of homogenate and dilutions were seeded in duplicate onto Baird Parker agar (BP, Merck) and incubated at 35 °C for 48 h. Black colonies surrounded by

an opaque zone and with an outer clear zone were selected for counting of *S. aureus*.

Characterization of *S. aureus*

Phenotypic characterization

The isolates of *S. aureus* were characterized as described previously (Satorres and Alcaráz, 2007). The interpretation of coagulase test was performed according to Sperberg and Tatini (1975). The 4+ reaction corresponded to a very firm opaque clot which remained in place when the tube was tipped on its side. The 2+ and 3+ reactions were not as opaque as 4+ reactions and were surrounded by clear plasma. The 1+ reaction showed a small and disorganized clot.

Simultaneously, an automated system (bioMérieux, Marcy l'Etoile, France) and a TECRA immunoassay equipment (International Pty Ltd, Australia) were used to determine the ability of *S. aureus* isolates to produce the pool of SEA, SEB, SEC1, SEC2, SEC3, SED, and SEE enterotoxins.

Genotypic characterization

For the detection of *sea*, *seb*, *sec*, *sed*, *see* and *16S rRNA* genes of *S. aureus*, a multiplex polymerase chain reaction (multiplex PCR) was used (Manfredi, 2007).

DNA extraction

Each strain was conserved in TSB (Britania) with 30% glycerol. From this culture medium each strain was spread on brain heart infusion agar (BHI, Becton Dickinson) and incubated for 24 h at 37 °C. After the incubation period, one colony was taken and inoculated in 5 mL of BHI broth, for 24 h at 37 °C. An aliquot of 1.5 mL was taken and centrifuged at 12000 x g for 5 min (Eppendorf 5415C). The supernatant was discarded and the pellet was resuspended in 150 µL of Triton X100 (1% in buffer TE 1X, 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8). The sus-

pension was boiled for 30 min and then centrifuged at 12,000 x g for 5 min to remove the bacterial debris. A 5-microliter aliquot was used as DNA template.

Multiplex PCR

The reaction mix had a final volume of 50 µL and contained the following compounds: 300 mM of each primer pair of the *sea*, *seb*, *sec*, *sed* and *see* genes (Table 1); 60 mM of the primer pair of *16S rRNA* gene (Invitrogen, Buenos Aires, Argentina) (Table 1); 400 µM of each dNTP (Promega; Madison, WI, USA); 1X PCR buffer (Fermentas, California, USA), 0.4 mM MgCl₂ (Fermentas), 0.04 U/µL of *Taq* polymerase (Fermentas), and 5 µL of DNA template. DNAs of enterotoxin producing *S. aureus* strains [M5 (*sea*), N14 (*seb*), MC1 (*sec*), C1 (*sed*), N19125 (*see*)], were used as positive controls, and the *S. aureus* ATCC 25923 strain was used as negative control. The reaction mixture without template was used as system control. The amplification conditions were: 95 °C for 5 min, followed by 15 cycles of 95 °C for 30 s, 68 °C for 30 s and 72 °C for 30 s; and 20 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s; with a final extension at 72 °C for 2 min.

A volume of 10 µL of a solution of xylene cyanol and glycerol 30% was added to 50 µL of amplified DNA, in 10 µL agarose gel 2.5% and 1X TAE buffer with 0.5 µg of bromide ethidium/mL. A 100 marker ladder (Biodynamics S. R. L., Buenos Aires, Argentina) was used. A model transilluminator 2000 (BioRad, Hercules, California, USA) and a C-5060 Wide Zoom Olympus (Melville, USA) were used for amplicons' observation. The analysis of the gels was performed with the Doc-It Image Acquisition software (UVP, Inc. Upland CA, USA) (Manfredi, 2007).

Antimicrobial susceptibility testing

The *S. aureus* strains were tested for susceptibility to a panel of antimicrobial agents using the disc diffusion

Table 1 - Primers used for the detection of staphylococcal enterotoxin genes.

Primers	Oligonucleotide sequence (5'-3')	Size of amplified product (bp)
sea F	GCA GGG AAC AGC TTT AGG C	521
sea R	GTT CTG TAG AAG TAT GAA ACA CG	
seb-sec F	ACA TGT AAT TTT GAT ATT CGC ACT G	667
seb R	TGC AGG CAT CAT GTC ATA CCA	
sec F	CTT GTA TGT ATG GAG GAA TAA CAA	284
sec R	TGC AGG CAT CAT ATC ATA CCA	
sed F	GTG GTG AAA TAG ATA GGA CTG C	385
sed R	ATA TGA AGG TGC TCT GTG G	
see F	TAC CAA TTA ACT TGT GGA TAG AC	171
see R	CTC TTT GCA CCT TAC CGC	
16S rRNA F	GTA GGT GGC AAG CGT TAT CC	228
16S rRNA R	CGC ACA TCA GCG TCA G	

method on Mueller-Hinton agar (MH, Merck) according to the Clinical and Laboratory Standards Institute (CLSI) (2007). The antibiotics tested were: penicillin G (PEN), 10 U; nitrofurantoin (NIT), 300 µg; oxacillin (OXA), 1 µg; erythromycin (ERY), 15 µg; gentamicin (GEN), 10 µg; rifampin (RIF), 5 µg; trimethoprim-sulfamethoxazole (TMS), 1.25 g/25 µg; vancomycin (VAN), 30 µg; ciprofloxacin (CIP), 5 µg; amikacin (AKN), 30 µg; fosfomicin (FOS), 50 µg and tetracycline (TET), 30 µg. The *S. aureus* ATCC 25923 strain was included in each trial as a control. This strain was cultured in BHI broth to 37 °C for 24 h and kept in Luria broth (LB; Merck) at -20 °C.

Inhibition assays

Chemicals

The inhibitory capacity of the following compounds, nisin (2.5% nisin with an activity of 1.020 IU/mg; Sigma-Aldrich), pure lysozyme from egg white (with an activity of 36.000 IU/mg; Fluka Chemie, Buchs, Switzerland) and EDTA (99% purity; Sigma-Aldrich), was assayed on *S. aureus* in contaminated fruit salads.

Minimum inhibitory concentration (MIC) assessment for *S. aureus*

The microtechnique of Gill and Holley (2003) was applied for determining the MIC of *S. aureus* corresponding to each chemical. The MIC values were expressed as mg/L.

Chemical treatments of *S. aureus* contaminated fruit salad

Taking into account the MIC values obtained as described, a study was conducted where a concentration equivalent to the MIC of each compound was added to 100 g of a sample of fruit salad. Each compound was tested alone or combining each other. Each sample was inoculated with 10⁵ cfu/mL of *S. aureus* ATCC 25923. The incubation conditions were 24 °C for 0, 4, 6, 24, 48 and 60 h. At each time, 1 mL of sample was taken and serial dilutions (1:10) in 0.1% PW pH 7 were performed. Then, 0.1 mL of each dilution was spread onto Baird Parker (BP; Britania), plates were incubated for 24 h at 37 °C and finally, counts of *S. aureus* colonies were done.

Samples without addition of any compound were treated under the same time and temperature conditions and used as control.

Statistical analysis

Survival was expressed as log₁₀ N – log₁₀ N₀, where N₀ was the initial *S. aureus* count in fruit salad and N was the bacterial count after antimicrobial treatment. Mean values of three replications were subjected to analysis of variance and Students test by Infostat 1.0 software for determining if significant variations (p < 0.05) in populations of microorganisms existed between treatments. The mean and

the standard deviation were calculated for the log₁₀ reduction of microorganism for each set of experiments.

Results

Microbiological analysis

The mean pH values observed in the fruit salads were 3.85 ± 0.24. The counts of total mesophilic aerobes ranged from 1.60 to 4.70 log₁₀ cfu/g (mean 3.25 ± 0.54). Total coliforms varied between 30 and 1.100 MPN/g; faecal coliforms were not detected. The counts of moulds and yeasts varied between 2.30 and 4.33 log₁₀ cfu/g (mean 3.17 ± 0.54). Enterobacteria such as *E. coli* O157:H7, *Salmonella* spp, *Shigella* spp, or *Y. enterocolitica* were not detected in any of the analysed samples.

S. aureus was isolated from eleven (7.81%) of the investigated samples, with counts varying between 1.30 and 2.47 log₁₀ cfu/g (mean 1.75 ± 0.49). All the isolates were thermonuclease positive and coagulase 4+. It was observed no organoleptic or physical difference between *S. aureus* positive and negative fruit salads. All salads were prepared with pieces of oranges, peaches, banana and apples, and had a similar pH. All *S. aureus* isolates showed positive results when the presence of *16S rRNA* gene was studied by multiplex PCR. Furthermore, 7 out of the 11 strains (63.6%) were positive for the *sea* gene (Figure 1) and for the production of the SEA enterotoxin. None of other investigated genes or toxins were detected. All strains were resistant to penicillin but susceptible to the remaining antimicrobial agents.

Inhibitory activity of antimicrobial compounds against *S. aureus*

The nisin MIC was 500 mg/L and the EDTA MIC was 250 mg/L at 24 h of incubation. Lysozyme did not show inhibitory capacity, either alone or combined with nisin. In fruit salads, with nisin at 60 h of incubation no inhibition was detected; meanwhile, with EDTA totally inhi-

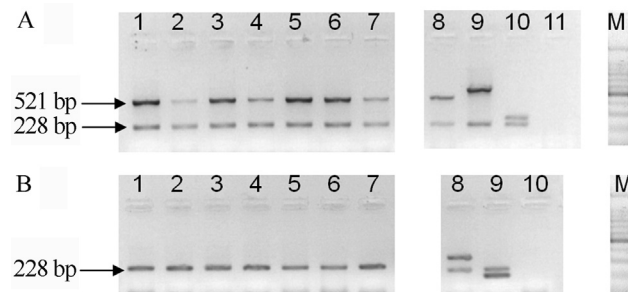


Figure 1 - Multiplex PCR for *S. aureus* enterotoxin genes. A) *sea* (521 bp), *seb* (667 bp), *sec* (284 bp), and *16S ARNr* (228 bp). Lines 1-7: isolates corresponded to the seven samples obtained from fruit salads; lines 8-10: *sea*, *seb* and *sec* positive controls respectively; line 11: negative control. B) *sed* (385 bp), *see* (171 bp), and *16S ARNr* (228 bp). Lines 1-7: isolates corresponding to the seven samples obtained from fruit salads; lines 8 and 9: *sed* and *see* positive controls respectively; line 10: negative control. M: 100 bp marker ladder.

bition of *S. aureus* at 60 h of incubation was observed (Figure 2).

Discussion

Over the last years, economic and job changes in Argentina have resulted in the spread of small manufacturers of different types of foods, particularly dairy products, fruit juices and their derivatives. Sometimes, these products are elaborated and sold by families or small groups of entrepreneurs under no optimal sanitary conditions. Fruit salads constitute a common product, usually packed in plastic containers and labelled with expiration date of generally 4 days after elaboration.

The Argentine Alimentary Code (AAC) in Article 969 provides specific microbiological recommendations for fruit salads (De la Canal, 2004). This food must be free of pathogenic microorganisms exhibiting the same microbiological quality than drinking water (mesophilic ≤ 500 cfu/mL, total coliforms ≤ 3 MPN/100 mL, and absence of faecal coliforms and *Pseudomonas aeruginosa*). In the present work, although no faecal coliforms were detected in the studied fruit salads, the range of mesophilic aerobic bacteria was between 1.60 and 4.70 \log_{10} cfu/g, and the values of total coliforms were between 30 and 1.100 MPN/g. The MPN/g of total coliforms in all the samples was higher than the AAC maximal limit for these microorganisms. The pH was 3.85 ± 0.24 , a value that can allow the growth of yeasts and moulds, which were between 2.30 and 4.33 \log_{10} cfu/g.

Many authors have studied the microbiological quality of fruit juices (Parish, 1997; Ghendhesh *et al.*, 2005), but very little literature is related to mixed fruit salads. Abadias *et al.* (2008) studied 21 kinds of fresh-cut fruit sold in streets of Catalonia, Spain, and reported that aerobic mesophilic bacteria ranged from 2.0 to 7.1 \log_{10} cfu/g, and yeast and mould counts were between 1.7 and 4.9 \log_{10} cfu/g, but they did not observe any pathogenic microorganisms. In a study of 38 fruit salad samples, among which eight were of mixed fruit, Tournas *et al.* (2006) reported that the yeast and moulds counts in the mixed fruit salads

ranged from 3.41 to 7.11 \log_{10} cfu/g, being higher than those observed in this work.

Many foods depend on a combination of a variety of factors like pH and low temperatures to extend their shelf life. Although the growth of many pathogenic microorganisms can be inhibited in such foods, they can survive for long periods at dangerous concentrations. In the present study, neither *Salmonella* spp, *Shigella* spp, *Y. enterocolitica* nor *E. coli* O157:H7 were detected in the fruit salads, but 11 strains of *S. aureus* were isolated. These results are in agreement with Kumar *et al.* (2006), who studied the microbiological characteristics of fruit salads sold in streets of Patiala, India, and detected coagulase positive *S. aureus* in 66 (44%) samples, 38 of these strains present produced enterotoxins B and C. Although the *S. aureus* concentration needed to produce food intoxication is high (10^5 - 10^6 cfu/g), this bacterium is able to grow at a wide range of pH (4.5-9.3) and temperature (7-43 °C) (World Health Organization, 1998). This could enable the growth of *S. aureus* in fruit salads during storage time and lead to health hazard for consumers. In the present work, the mean count of *S. aureus* was $1.75 \pm 0.49 \log_{10}$ cfu/g. Among the 11 strains analysed for the presence of enterotoxin genes by multiplex PCR, seven were positive to *sea* gene. In fact, enterotoxin A together with the toxic shock syndrome toxin-1 (TTSS) were the most frequently found toxins in *S. aureus* isolates from food-poisoning cases in Taiwan (Chiang *et al.*, 2008). The *sea* gene coding for enterotoxin A has also been frequently detected in *S. aureus* strains isolated from foods (Kerouanton *et al.*, 2007; López *et al.*, 2008; Rall *et al.*, 2008).

Three different antimicrobial components were tested to evaluate their inhibitory activity against *S. aureus*. In our work, at the initial 24 h of nisin action, *S. aureus* was sensitive to 500 mg/mL; however, at 60 h of incubation no concentration of nisin inhibited the growth of this bacterium. Our results are similar to those obtained by Lima Grisi and Goralach-Lira (2005), who observed a significant inhibition of *S. aureus* in pure culture for all nisin concentrations (100-1200 $\mu\text{g/mL}$) used, but only for 8 h of incubation. The inhibition phase was followed by a period of rapid growth of the strain, achieving levels similar to those of the control after 24 h. Piper *et al.* (2009) studied 55 *S. aureus* strains and reported that nisin was active against all the assayed strains with a MIC between 0.5 to 8.3 mg/L. Microorganisms exhibiting resistance to nisin may act by different ways, inactivating the peptide via enzymatic action, altering membrane susceptibility (Montville and Chen, 1998), or producing the enzyme nisinase that neutralizes the antimicrobial activity of the polypeptide (Hurst and Hoover, 1993). In the present study, lysozyme did not inhibit the growth of *S. aureus* neither in pure culture nor in fruit salads. Bera *et al.* (2007) have shown that modifications in peptidoglycan (PG) by O acetylation, wall teichoic acids,

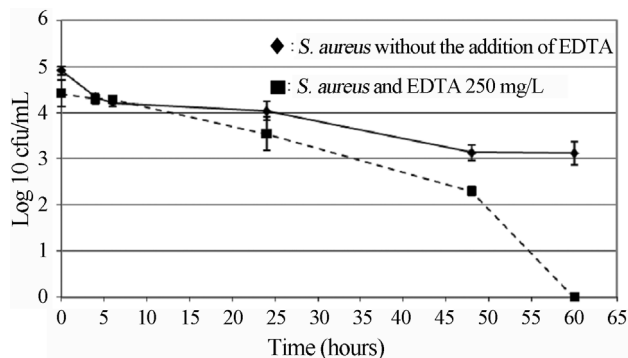


Figure 2 - Activity of EDTA against *S. aureus* inoculated in artisan fruit salads.

and a high degree of cross-linking lead to the loss of sensitivity to lysozyme by *S. aureus*.

The obvious implication of the emergence of pathogenic microorganisms resistant to bacteriocins is the potential risk in foods that are preserved exclusively by a single compound. To overcome this hazard, some researchers have suggested using combinations of bacteriocins with another antimicrobial. In theory, combinations of bacteriocins could be successfully applied if the mechanisms of action of the bacteriocins were different (Chung and Hancock, 2000). In our case, such interaction was not observed when nisin was combined with lysozyme.

EDTA produced a total inhibition of *S. aureus* growth at 60 h of incubation in a concentration of 250 mg/L, both in broth and in fruit salads. These results agree with those of other authors who observed a complete inhibitory effect of EDTA, in broth medium, against several microorganisms: *E. coli*, *Enterococcus faecalis*, *Shewanella putrefaciens* and *S. Enteritidis*, among other bacteria (Boziaris and Adams, 1999; Gill and Holley, 2003).

Although the artisan fruit salads studied in the present work were purchased from different shops, all *S. aureus* strains were resistant to penicillin and susceptible to the other tested antimicrobial agents. K erouanton *et al.* (2007) reported that only two strains of 178 analysed in France were susceptible to all tested agents whereas 63.6% of the strains were resistant to three or more agents.

The presence of *S. aureus* in artisan fruit salads could indicate that the hygienic conditions during the elaboration of these products were inadequate. Moreover, the presence of strains carrying the *sea* gene detected in our work increases the risk of intoxication for consumers of fruit salads. Although nowadays no additives are added in the artisan products that are commercialized in our city, our results show that EDTA could be useful to extend the shelf life of fruit salads and decrease microorganism number. Furthermore, the obtained results highlight the need of sanitary education led to food handlers for arising awareness and improving the hygienic conditions by preparing foods.

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