

# 1 Incidence and characterization of *Staphylococcus aureus* in 2 fishery products marketed in Galicia (Northwest Spain)

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14

1   **Abstract**

2   A total of 298 fishery products purchased from retail outlets in Galicia (NW Spain) between  
3   January 2008 and May 2009 were analyzed for the presence of *Staphylococcus aureus*. *S.*  
4   *aureus* was detected in a significant proportion of products (~25%). Incidence was highest in  
5   fresh (43%) and frozen products (30%), but it was high in all other categories: salted fish  
6   (27%), smoked fish (26%), ready-to-cook products (25%), non-frozen surimis (20%), fish  
7   roes (17%) and other ready-to-eat products (10%). A significant proportion of smoked fish,  
8   surimis, fish roes and other ready-to-eat products did not comply with legal limits in force.

9   RAPD-PCR of 125 *S. aureus* isolated from fishery products was carried out using three  
10   primers (AP-7, ERIC-2 and S). Isolates displayed 33 fingerprint patterns. Each pattern was  
11   attributed to a single bacterial clone. Cluster analysis based on similarity values between  
12   RAPD fingerprints did not find relationship between any RAPD pattern and any product  
13   category.

14   Isolates were also tested for *se* genes and susceptibility to a range of antibiotics (cephalothin,  
15   clindamycin, chloramphenicol, erythromycin, gentamicin, oxacillin, penicillin G, tetracycline,  
16   vancomycin, methicillin, ciprofloxacin and trimethoprim-sulfamethoxazole). Most isolates  
17   (88%) were found to be *sea* positive. Putative enterotoxigenic strains counts reached high risk  
18   levels in 17 products. No relationship was found between the presence of *se* genes and RAPD  
19   patterns. All isolates were resistant to penicillin, chloramphenicol and ciprofloxacin, and most  
20   to tetracycline (82.4%), but none was methicillin-resistant.

21   A revision of pre-requisite programs leading to improve hygienic practices in handling and  
22   processing operations from fishing or farming to retail is recommended to ensure fishery  
23   products safety.

1   Keywords: *Staphylococcus aureus*; *fishery products*; *retail level*; *enterotoxin genes*;

2   *antibiotic resistance.*

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## 1   **1. Introduction**

2   Although it is necessary to ensure food safety for the health of consumers and industry,  
3   *Salmonella* spp., *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus* and  
4   pathogenic vibrio species have been repeatedly detected in a diverse variety of fishery  
5   products (EFSA, 2010; Garrido et al., 2009; Herrera et al., 2006; Kumar et al., 2009; Novotny  
6   et al., 2004; Papadopoulou et al., 2007; Yang et al., 2008). Novel trends in food production  
7   such as minimal processing, mass production and globalization, among others, have  
8   additionally introduced new factors and conditions that can enhance the presence and  
9   subsequent growth of bacterial pathogens (Abee and Wouters, 1999; Cebrián et al., 2007;  
10   Rendueles et al., 2011).

11   *S. aureus* is one of the major bacterial agents causing foodborne diseases in humans  
12   worldwide (EFSA, 2010; Le-Loir et al., 2003). Staphylococcal food poisoning is usually self-  
13   limiting and resolves within 24 to 48 h after onset. Most cases are therefore not reported to  
14   healthcare services. As a result, the actual incidence of staphylococcal food poisoning is  
15   known to be much higher than reported (Lawrynowicz-Paciorek et al., 2007; Smyth et al.,  
16   2004). In addition, the notification of staphylococcal intoxications is not mandatory in a  
17   number of member states of the European Union. Staphylococcal food poisonings result from  
18   the ingestion of food containing staphylococcal enterotoxins (SEs) preformed by  
19   enterotoxigenic strains (Kérouanton et al., 2007; Le-Loir et al., 2003). SEs are resistant to  
20   proteolysis and heat-stable, so the presence of SEs involves a significant food safety risk  
21   (Omoe et al., 2005).

22   The widespread use of antibiotics has evolved the emergence of multi-drug resistant strains,  
23   and it makes eradication more difficult and incidence to increase. Multi-resistant *S. aureus* is  
24   rather common in hospital settings and farms (Livermore, 2000; Sakoulas and Moellering,

1 2008). Community-associated multi-resistant *S. aureus* is becoming an emerging problem too  
2 (Popovich et al., 2007; Ribeiro et al., 2007; Stankovic et al., 2007). Antibiotic-resistant strains  
3 of *S. aureus* have been detected in food animals (Lee, 2003) and food like meat (Normanno et  
4 al., 2007; Pesavento et al., 2007), milk and dairy products (Gündogân et al., 2006; Peles et al.,  
5 2007; Pereira et al., 2009) and also fishery products (Beleneva, 2011), and it may be very  
6 hazardous for human health.

7 The identification of bacterial clones with enhanced virulence or increased ability to spread is  
8 important. Nowadays, PCR-based techniques are commonly used for typing, as they are easy,  
9 fast and cost-effective. Among such techniques, random amplified polymorphic DNA  
10 (RAPD-PCR) has been considered a very useful tool for rapid differentiation of clones with  
11 no prior information of the gene sequence (Fueyo et al., 2001; Nema et al., 2007; Nikbakht et  
12 al., 2008; Shehata, 2008; Van-Belkum et al., 1995).

13 Nowadays, Spain is the largest fishery producer, particularly in Galicia (NW Spain), and the  
14 second largest consumer in the European Union (Eurostat, 2007). However, no results have  
15 been found on the incidence of bacterial pathogens in fishery products made or sold in  
16 Galicia, apart from one study on molluscan shellfish farmed in Galician waters (Martínez et  
17 al., 2009). The situation is not different for fishery products marketed in other parts of Spain,  
18 and only two studies on smoked fish (Garrido et al., 2006; Herrera et al., 2006) and another  
19 study on freshwater fish (González-Rodríguez et al., 2002) have been carried out in the last  
20 decade.

21 Therefore, the present study was aimed to determine the incidence of *S. aureus* in fishery  
22 products marketed in Galicia and subsequently identify most common clones by RAPD-PCR,  
23 as well as cases of increased risk according to the presence of enterotoxin genes and  
24 antibiotic-resistance of isolates.

1      **2. Materials and Methods**

2      *2.1. Sampling*

3      A total of 298 fishery products marketed at different retail outlets in Vigo (Galicia, Northwest  
4      Spain) were purchased and analyzed between January 2008 and April 2009. Fourteen  
5      samplings (approximately one each month) were carried out. Products were classified into  
6      eight different categories: fresh products, frozen products, salted fish, ready-to-cook products,  
7      smoked fish, fish roes, non-frozen surimis and other ready-to-eat products (seafood salads,  
8      pâtés and anchovies in oil). Between 24 and 43 products of each category were analyzed.

9      *2.2. Isolation and identification of *S. aureus**

10     About 50 g of product mixed with 200 ml of peptone water was homogenized in a stomacher  
11     masticator (IUL instruments, Barcelona, Spain). Subsequently, homogenates were serially  
12     diluted in peptone water (1:50 and 1:500). Aliquots (0.5 ml) of each dilution spread onto  
13     Baird Parker agar supplemented with egg yolk tellurite emulsion (Biolife, Milan, Italy) (BP-  
14     EY). Plates were incubated at 37°C for 48 h.

15     Typical colonies of *S. aureus* as well as non-typical colonies (showing no white margin and  
16     smaller than 2 mm) were counted. Between 1 and 9 colonies from each product were selected  
17     and sub-cultured twice on BP-EY agar for isolation of single colonies (isolates).

18     Isolates cultured in Brain Heart Infusion broth (Biolife) (BHI) for 24 h at 37°C were subjected  
19     to three different biochemical tests: coagulase, DNase and mannitol fermentation.

20     Coagulase production was tested by adding 100 µl of bacterial culture into 300 µl of  
21     reconstituted rabbit plasma with EDTA (Bactident® Coagulase rabbit, Merck, Darmstadt,  
22     Germany) followed by incubation of tubes at 37°C. Clotting of plasma was assessed at 1-h  
23     intervals during 6 h and after 24 h of incubation.

1 Isolates were streaked onto DNase agar (Cultimed, Panreac Quimica, Barcelona, Spain)  
2 supplemented with D-mannitol and bromothymol blue and plates were incubated at 37°C for  
3 24 h. The surface of the plates was then flooded with 0.1 N HCl during 15-20 min for DNA  
4 precipitation. DNase activity was observed by the presence of a transparent halo around the  
5 colonies on the agar. Mannitol fermentation was observed as a colour change of the pH  
6 indicator -from blue to yellow- due to acid production.

7 Colonies found to be coagulase positive, DNase positive and able to ferment mannitol  
8 (suspected *S. aureus*) were confirmed to be *S. aureus* by species-specific 23S rDNA PCR.  
9 Genomic DNA was extracted from 24 h cultures in BHI using an InstaGene™ Matrix kit  
10 (Bio-Rad Laboratories, S.A., Madrid, Spain) following manufacturer's instructions. DNA was  
11 quantified by assuming that an absorbance value at 260 nm of 0.100 corresponds to 5 µg/ml  
12 of DNA. Primers staur4 (5'-ACGGAGTTACAAAGGACGAC-3') and staur6 (5'-  
13 AGCTCAGCCTAACGAGTAC-3') (Straub et al., 1999) were used for each strain.  
14 Expected size of amplified PCR products was 1250 bp. Each PCR mixture contained 100 ng  
15 DNA, 1x Taq Buffer Advanced, 2.5 U Taq DNA polymerase (5 Prime, Hamburg, Germany),  
16 40 nmol of each dNTP (Bioline, London, United Kingdom), 0.25 nmol of forward and reverse  
17 primers (Thermo Fisher Scientific, Ulm, Germany) and sterile Milli-Q water up to a final  
18 volume of 50 µl. PCR was performed with a MyCycler™ Thermocycler (Bio-Rad). The  
19 conditions proposed by Vautor et al. (2008) were used to target the 23S rDNA gen. An initial  
20 step of 5 min at 94°C was followed by 30 cycles of 30 s at 94°C, 30 s at 58°C and 75 s at  
21 72°C, and a final step at 72°C for 5 min. PCR products were subjected to electrophoresis on  
22 1.5% agarose gel containing ethidium bromide for 90 min at 75 V and 100 mAmp. Gels were  
23 photographed in a Gel Doc XR system (Bio-Rad) using the Quantity One® software (Bio-  
24 Rad). A DNA ladder of 50-2000 bp (Hyperladder II, Bioline) was included as a molecular  
25 size marker.

1 Stock cultures of *S. aureus* isolates were maintained in 50% glycerol (w/w) at -80 °C. When  
2 needed, stock cultures were thawed and subcultured twice in tryptic soy broth (Cultimed) for  
3 24 h at 37 °C prior to being used.

4 **2.1. RAPD**

5 Genotypic characterization of isolates was performed by RAPD-PCR. DNA was extracted  
6 and quantified as previously described from two different cultures of each isolate to check  
7 reproducibility of banding profiles. Primers S (5'-TCACGATGCA-3') (Martín et al., 2004),  
8 AP-7 (5'-GTGGATGCGA-3') and ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGCG-3')  
9 (Van-Belkum et al., 1995) were individually used in separate reactions with each isolate. Each  
10 PCR mixture consisted of 200 ng DNA; 1x Taq Buffer Advanced and 2.5 U Taq DNA  
11 polymerase (5 Prime); 40 nmol of each dNTP (Bioline); 0.25 nmol primer (Thermo Fisher  
12 Scientific) and sterile Milli-Q water up to a final volume of 50 µl. PCR mixtures with primers  
13 AP-7 and ERIC-2 were further supplemented with 1mM MgCl<sub>2</sub> (5 Prime). RAPD-PCR was  
14 performed with a MyCycler™ Thermocycler (Bio-Rad). PCRs containing primer S consisted  
15 of an initial cycle at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 37°C for 1 min  
16 and 72°C for 2 min, with a final extension of 5 min at 72°C. Amplification conditions for AP-  
17 7 and ERIC-2 included a denaturation cycle at 94°C for 4 min, 35 cycles of 94°C for 1 min,  
18 25°C for 1 min and 72°C for 2 min, and a last extension at 72°C for 7 min. PCR products  
19 were subjected to electrophoresis on 1.5% agarose gel containing ethidium bromide as  
20 aforementioned. A DNA ladder of 50-2000 bp was included in all gels.

21 A second-order polynomial relationship between molecular size and mobility was obtained  
22 for each gel ( $r > 0.99$ ) and used to determine the molecular size of DNA bands. A RAPD  
23 pattern was described as different when at least one band difference was found.  
24 Reproducibility of patterns was checked twice using independent DNA samples. Variations in  
25 band intensity were not considered. Bands too faint to be reproduced were not considered. A

1 binary value (0 or 1) denoting absence or presence of each band was assigned to each pattern.  
2 Similarity analysis determining the Dice coefficients (Struelens et al., 1996) was performed  
3 by IBM SPSS 19.0. Cluster analysis by UPGMA (Sneath and Sokal, 1973) and dendograms  
4 were performed with StatistiXL 1.8.

5 *2.2. Detection of sea-see and seg-sei genes*

6 A slight modification of the method described by Omoe et al. (2002) was followed to analyze  
7 the presence of staphylococcal enterotoxin (*se*) genes. Two multiplex PCR detecting *sea-see*  
8 and *seg-sei* genes were performed for each strain. DNA was extracted and quantified as  
9 previously described. Primer nucleotide sequences and expected sizes of amplicons are shown  
10 in Table 1. Each PCR mixture contained 100 ng DNA; 10x KCl reaction buffer and 40 nmol  
11 of each dNTP (Bioline); 40 pmol SEC-3/SEC-4 primers, 80 pmol SEB-1/SEB-4 primers and  
12 20 pmol for other primers (Thermo Fisher Scientific); 2.5 U Taq DNA polymerase (5 Prime)  
13 and sterile Milli-Q water up to a final volume of 50 µl. *S. aureus* ATCC 12600 was used as a  
14 negative control in all PCRs, whereas *S. aureus* ATCC 13565 was used as a positive control  
15 for *sea* and *sed*, *S. aureus* ATCC 19095 for *sec*, *seg*, *seh* and *sei*, *S. aureus* ATCC 14458 for  
16 *seb*, and *S. aureus* ATCC 27664 for *see*. All these strains were obtained from the Spanish  
17 Type Culture Collection (CECT). All PCRs were performed with a MyCycler™  
18 Thermocycler (Bio-Rad) as follows: an initial cycle of 95°C for 2 min, 55°C for 1 min and  
19 68°C for 2 min, followed by 28 cycles of 95°C for 1 min, 55°C for 1 min and 68°C for 2 min,  
20 and a final cycle of 95°C at 1 min, 55°C for 1 min and 68°C for 5 min. PCR products were  
21 subjected to electrophoresis on 2.5% agarose gel containing ethidium bromide. Run  
22 conditions and gel display were as aforementioned. A DNA ladder of 50-2000 bp was also  
23 included in all gels.

24 *2.3. Antibiotic susceptibility test*

1 Minimal inhibitory concentration (MIC) of twelve antibiotics was determined against *S.*  
2 *aureus* isolated. The EUCAST guidelines (2003) for broth microdilution and disk diffusion  
3 testing were followed.

4 Broth microdilution test was performed with nine antibiotics: cephalothin, oxacillin, penicillin  
5 G and vancomycin (Sigma-Aldrich Química, Madrid, Spain); clindamycin and erythromycin  
6 (Acofarma, Barcelona, Spain); and chloramphenicol, gentamicin and tetracycline (Fagron  
7 Iberica, Terrasa, Spain). Adjusted cultures of each isolate to  $5 \cdot 10^5$  CFU/ml in Muller Hinton  
8 broth (Cultimed) supplemented with  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (25 mg/ml) and  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (12.5 mg/ml)  
9 were exposed to each antibiotic into a microtiter plate (Falcon<sup>®</sup>, Becton Dickinson Labware,  
10 USA) for 18-20 h (or 24h for oxacillin and vancomycin) at 35°C. The OD<sub>655nm</sub> was measured  
11 in an iMark Microplate Reader through Microplate Manager 6<sup>®</sup> software (Bio-Rad). MIC was  
12 defined as the minimum antibiotic concentration at which no growth was observed.

13 The susceptibility of isolates to methicillin (Oxoid, Basingstoke, United Kingdom),  
14 ciprofloxacin and trimethoprim-sulfamethoxazole (bioMerieux España, Madrid, Spain) was  
15 determined by a disk diffusion test. Adjusted cultures were evenly spread on Muller Hinton  
16 agar (Cultimed) and commercially prepared antibiotic disks were placed on the agar surface.  
17 The length of the inhibition halo was measured after 16-18 h (24 h for methicillin) at 35°C.

18 The reference strains *S. aureus* ATCC 29213 and *S. aureus* ATCC 43300 purchased from the  
19 CECT was used in all these tests as negative control and positive control respectively.  
20 Antibiotic susceptibility was classified as sensitive, intermediate or resistant on the basis of  
21 the breakpoints reported in Table 2.

22 *2.4. Detection of blaZ and meca genes*

23 A slight modification of the methods described by Baddour et al. (2007) and Olsen et al.  
24 (2006) was followed for detecting genes encoding penicillin (*blaZ*) and methicillin resistance

1 (*mecA*), respectively. DNA was extracted with DNeasy® kit (Qiagen, Hilden, Germany)  
2 according to the manufacturer. Extraction was tested by using  $\lambda$  *Hind*III DNA Ladder as a  
3 reference (564-23130 bp) (New England BioLabs™, Ipswich, USA). Primers blaZF487 (5'-  
4 TAAGAGATTGCCTATGCTT-3') and blaZR373 (5'-TTAAAGTCTTACCGAAAGCAG-  
5 3') for *blaZ* gen, and *mecA*1-F (5'-TGGCTATCGTGTACAATCG-3') and *mecA*2-R (5'-  
6 CTGGAACTTGTTGAGCAGAG-3') for *mecA* gen, were used. Expected sizes of amplified  
7 PCR products were 377 bp for *blaZ* gen and 309 bp for *mecA* gen. PCR mixtures were  
8 composed of 20 ng of DNA; 5 nmol of each dNTP (Invitrogen Corporation, Carlsbad, USA);  
9 2.5  $\mu$ l of Dynazym buffer 10x and 1.2 U of Dynazym Hot Start (Bio-Rad); 10 pmol of  
10 forward and reverse primer and sterile Milli-Q water up to a final volume of 25  $\mu$ l. PCRs were  
11 performed with an 80 Gene Amp PCR System 9700 (Applied Biosystems, Carlsbad, USA).  
12 For *mecA* gen, PCR consisted of an initial denaturation at 94°C for 5 min, followed by 30  
13 cycles at 94°C for 1 min, 54°C for 1 min and 72°C for 1 min, and a final cycle at 72°C for 7  
14 min. Conditions for *blaZ* gen detection consisted of denaturation at 94°C for 5 min, 35 cycles  
15 of 94°C for 1 min, 54°C for 1 min and 72°C for 1 min, and a last extension at 72°C for 10 min.  
16 *S. aureus* ATCC 29213 was used as negative control, whereas *S. aureus* ATCC 43300 was  
17 used as positive control. Amplicons were subjected to electrophoresis on 1.2% agarose gel  
18 containing ethidium bromide for 30 min at 100 V and 200 mAmp. Gels were visualized and  
19 saved in a Typhoon Scanner 8600 (Molecular Dynamics, GE Healthcare, Little Chalfont,  
20 UK). A DNA ladder of 154-2176 bp (DNA Molecular Weight Marker VI, Roche Applied  
21 Science, Indianapolis, USA) was included in all gels.

22

1    **3. Results**

2    *3.1. Incidence in fishery products*

3    Colonies were observed on BP-EY for 167 out of 298 fishery products. A total of 728  
4    colonies were picked up, isolated and subjected to phenotypic (coagulase, DNase and  
5    mannitol fermentation) confirmation tests. Out of them, 125 were positive by all tests and thus  
6    identified as *S. aureus*. Additionally, one representative isolate of each RAPD global pattern  
7    (see below) was analyzed by species-specific 23S rDNA PCR. All of them were confirmed as  
8    *S. aureus*. These isolates were obtained from 75 fishery products, which represented an  
9    incidence of 25.16%.

10   As shown in Figure 1, incidence was different in each product category, and it decreased in  
11   the following order: fresh products (43%), frozen products (30%), salted fish (27%), smoked  
12   fish (26%), ready-to-cook products (25%), non-frozen surimis (20%), fish roes (17%), and  
13   lastly other ready-to-eat products (10%).

14   A significant proportion of surimis (9%), fish roes (4%) and other ready-to-eat products (5%)  
15   as well as ready-to-cook products (13%) exceeded  $10^2$  CFU/g of food, which was the  
16   maximum number of *S. aureus* allowed by legislation in force when the study was conducted  
17   (O. 2/8/1991, RD 3484/2000 and Commission Regulation (EC) No 2073/2005). Additionally,  
18   counts were higher than  $10^3$  CFU/g in 9 out of 35 surimis, 5 out 41 other ready-to-eat  
19   products and 8 out of 40 ready-to-cook products. In the same way, *S. aureus* must have been  
20   absent in anchovies in oil, but it was detected in 2 out of 4 products.

21   The present results have also shown that a significant proportion (18.6%) of smoked products  
22   exceeded the limit set for smoked fish products in O. 2/8/1991, that is,  $2 \cdot 10^1$  CFU/g of food.  
23   Later, Commission Recommendation 2001/337/EC proposed that counts higher than  $10^2$   
24   CFU/g (M value) should not be permitted in smoked fish. The value set for M value was

1 exceeded in 16.3% of the smoked products tested in this study. Additionally, counts were  
2 higher than exceeded  $10^3$  CFU/g of food in 3 out of 43 smoked products (7%).

3 Although it is not subject to legal regulations, it is also worthy to mention that the number of  
4 *S. aureus* was higher than  $10^2$  CFU/g of food in 19.4% of fresh products, 14% of frozen  
5 products and 13.3% of salted fish, and  $10^3$  CFU/g of food in 7% of frozen products, 4.8% of  
6 fresh products and 6.7% of salted fish.

7 **3.2. RAPD-PCR**

8 Genotypic characterization of isolates was performed by RAPD-PCR with three different  
9 primers (S, AP-7 and ERIC-2). As shown in Figure 2, RAPD analysis with primer S yielded  
10 13 visually different banding profiles, whereas 12 profiles were obtained with each of the two  
11 other primers. A total of 31 different bands with a size between 115 and 2292 bp were  
12 amplified by primer S, whereas primer AP-7 and ERIC-2 amplified 19 and 18 different bands  
13 ranging from 127 to 2023 bp and 125 to 1420 bp, respectively. A good reproducibility of  
14 patterns was achieved when DNA from different cultures of each isolate was used as a  
15 template.

16 The combination of RAPD fingerprints obtained in separate reactions with different primers  
17 has been employed as a strategy to increase the discriminatory power of the analysis (Byun et  
18 al., 1997; Nema et al., 2007). The combination of the patterns obtained with the three primers  
19 generated a higher discriminatory power ( $D=0.926$ ) than those of single primers (0.818 for  
20 primer S, 0.698 for ERIC-2 and 0.622 for AP-7) as well as of pairwise-combinations of  
21 primers (0.883 for S and ERIC-2, 0.878 for S and AP-7 and 0.850 for AP-7 and ERIC-2). As  
22 a result, 33 combined or global fingerprints were distinguished. A three-digit code number  
23 were assigned to combined patterns, each one corresponding to the number assigned to  
24 patterns obtained with primers S, AP-7 and ERIC-2, respectively. The most common

1 combined pattern was 4.4.1., which was characteristic of 23 isolates. Also, two other patterns  
2 (1.1.1 and 13.1.11) were shared by 12 and 16 isolates, respectively. Thus, 40.8% of isolates  
3 were included in one of these three patterns. In contrast, 18 patterns were specific to one  
4 isolate. Isolates showing identical combined RAPD-PCR fingerprints were considered to be a  
5 single genetic type, that is, a single bacterial clone or strain (Fueyo et al., 2001). Thus, strains  
6 St.1.10 and St.1.31 were the most prevalent in fishery products, being found in 15 and 16  
7 products, respectively.

8 Cluster analyses based on similarity measurements from RAPD fingerprints were carried out  
9 with the aim of finding possible relationships between RAPD patterns and product categories.  
10 Cluster analysis of combined RAPD patterns classified isolates into 17 groups at a relative  
11 genetic similarity of 0.84 (Figure 3). Clusters 1 and 2 were the largest ones and contained 4  
12 and 11 patterns, respectively, which included most isolates (31 and 57, respectively) from all  
13 product categories. In contrast, there were 10 single clusters which were formed by only one  
14 isolate. The other two single clusters (13.5.11 and 9.4.7) were composed of 3 and 4 isolates.

15 The validity assessment of cluster analyses rendered low values of hierarchical F-measures  
16 for banding patterns generated with primers S (0.314), ERIC-2 (0.311) and AP-7 (0.289).  
17 Similarly, low values were also obtained for F-measure, precision and recall in all cases.  
18 Hierarchical F-measure did not increase when banding patterns generated with each primer  
19 were combined (0.329). Values of F-measure, precision and recall did not increase in this case  
20 either. This assessment showed that no cluster was relatively pure and included most isolates  
21 of only one product category. No relationship was therefore found between any RAPD pattern  
22 and any product category.

23 *3.3. Presence of enterotoxin genes*

1 Over 91% of *S. aureus* isolates (n=114) carried enterotoxin genes, from which 112 were *sea*  
2 positive. However, only four isolates carried several enterotoxin genes. In two isolates, the  
3 *seg* and *sei* genes were detected, whereas two others carried the *sea*, *sec* and *seh* genes. Each  
4 of these isolates shared a different combined RAPD pattern, so they were different bacterial  
5 strains. Nevertheless, no further relationship was found between RAPD patterns of *se-*  
6 carrying isolates.

7 All isolates sharing an identical global RAPD pattern carried the same *se* gen pattern, and this  
8 supports the thesis that they are bacterial clones. A total of 26 out of the 33 strains identified  
9 by RAPD-PCR analysis were *se* gene carriers. *S. aureus* St.1.10 and St.1.31, which were most  
10 prevalent, were *se* positive. No *se*-carrying strain was characteristic of a single product  
11 category.

12 Non-*se*-carrying strains showed distinct RAPD fingerprints, which were not shared by any *se-*  
13 carrying strain. However, no further relationship was found between the presence of *se* genes  
14 and any RAPD pattern. Cluster analysis did not discriminate any cluster comprising either all  
15 non-*se*-carrying strains or all multi-*se*-carrying strains only.

16 A significant proportion of the fishery products tested (23.5%) were contaminated with *se-*  
17 positive *S. aureus*. Furthermore, counts of *se*-carrying *S. aureus* exceeded  $10^2$  and  $10^3$  CFU/g  
18 of food in 34 and 18 products, respectively, and were even higher than  $10^5$  CFU/g of food in  
19 two dried-salted tuna loin products (i.e. mojama) and one surimi product.

20 The incidence of *se*-positive *S. aureus* was different in each product category (Figure 4).  
21 Isolates carrying *se* genes were found in all salted fish (n=8), surimis (n=7) and fish roes  
22 (n=4) which were contaminated with *S. aureus*. The presence of *se*-positive isolates was also  
23 very high in fresh products (94%), smoked fish (91%), ready-to-cook products (90%), frozen  
24 products (85%) and other ready-to-eat products (75%) in which *S. aureus* was detected.

1 The presence of *se*-positive and *se*-negative isolates was not detected in a same fishery  
2 product, except in one frozen hake nuggets product and one fresh perch fillet, in which both  
3 types were found. Moreover, one cod pâté with pepper carried *sea* positive and *sea*, *sec* and  
4 *seh* positive isolates.

5 *3.4. Antibiotic sensitivity*

6 No differences were found among isolates with regard to sensitivity profiles, except for  
7 tetracycline. All strains were thus found to be resistant to penicillin G, chloramphenicol and  
8 ciprofloxacin. However, differences were found with regard to MIC values. Thus, strains  
9 St.1.11 and St.1.18 were the most resistant to penicillin G, St.1.01 and St.1.31 were the most  
10 resistant to chloramphenicol and St.1.26 and St.1.33 showed the highest MIC value (1.58  
11 µg/ml) for ciprofloxacin. On the contrary, none of the strains was found to be resistant to  
12 beta-lactam antibiotics such as oxacillin and methicillin, but they all had intermediate  
13 resistance to methicillin (MIC from 9.5 to 14.5 µg/ml). Strains St.1.11 and St.1.13 showed the  
14 highest levels of resistance to methicillin (MIC=14.5 µg/ml). No strain was resistant to  
15 vancomycin, cephalothin, clindamycin, erythromycin, gentamicin or trimethoprim-  
16 sulfamethoxazole.

17 Tetracycline was the only antibiotic on which both resistant- and sensitive-strains were found.  
18 Thus, 17 out of the 33 strains (51.5%) identified by RAPD-PCR analysis were tetracycline-  
19 resistant, being *S. aureus* St.1.20 and St.1.31 the most resistant. However, over 82% of  
20 isolates were tetracycline-resistant. Tetracycline-resistant isolates were detected in 65 out of  
21 75 fishery products contaminated with *S. aureus* (86.7%). St.1.10 and St.1.31, which were  
22 most prevalent, were also tetracycline-resistant. No differences in tetracycline susceptibility  
23 of isolates sharing identical global RAPD patterns with all three primers were found.  
24 However, no relationship was found between any RAPD pattern and susceptibility to  
25 tetracycline.

1 The incidence of tetracycline-resistant *S. aureus* was different in each product category  
2 (Figure 5). Tetracycline-resistant isolates were detected in all salted fish and fish roes which  
3 were found to be contaminated with *S. aureus*. Tetracycline-resistant isolates were also found  
4 in most contaminated fishery products of other categories, with an incidence decreasing in the  
5 following order: surimis (93.7%), ready-to-cook products (87.5%), smoked fish (82.4%),  
6 other ready-to-eat products (80.0%), fresh products (76.0%) and frozen products (64.3%).

7 Additionally, one representative isolate of each *se*-carrying strain was tested for the presence  
8 of *blaZ* and *mecA* genes, which are major determinants of the resistance of staphylococci to  
9 penicillin (Olsen et al., 2006; Vesterholm-Nielsen et al., 1999) and methicillin and all other  
10 beta-lactam antibiotics (Baddour et al., 2007; Strommenger et al., 2003), respectively. No  
11 PCR product was detected for *mecA* in any of the 26 isolates tested, whereas *blaZ* was  
12 detected in all of them (data not shown). These results are in agreement with those of  
13 antibiotic sensitivity testing.

14

1    **Discussion**

2    A high incidence of *S. aureus* was found in fishery products marketed in Galicia (25.16%) in  
3    the present study. Although data on the incidence of *S. aureus* in fishery products was scarce,  
4    a high incidence had also been reported over the last ten years in some studies (Abrahim et  
5    al., 2010; Herrera et al., 2006; Oh et al., 2007; Papadopoulou et al., 2007; Simon and Sanjeev,  
6    2007), and only one work on fishery products collected at retail outlets in Italy found a low  
7    incidence, i.e. < 3% (Normanno et al., 2005). Only a few studies on microbial safety of fish  
8    products marketed in Spain have been carried out in the last decade (Garrido et al., 2009;  
9    González-Rodríguez et al., 2002; Herrera et al., 2006; Martínez et al., 2009). Considering the  
10   importance of fishery products at national level, the lack of information available on  
11   microbial safety in fishery products made or sold in Spain, and particularly in Galicia, was  
12   found surprising. Only Martínez et al. (2009) tested for the presence of bacterial pathogens in  
13   fishery products made in Galicia, specifically in farmed molluscan shellfish.

14   A wide range of product categories has been examined in the present work, comprising  
15   between 24 and 43 products of each one. In contrast, previous studies on the incidence of *S.*  
16   *aureus* in fish products have focused in only one or two product categories. Thus, Da-Silva et  
17   al. (2010), Herrera et al. (2006), Oh et al. (2007) and Papadopoulou et al. (2007) only tested  
18   fresh fishery products obtained from retail stores in Brazil, Spain, Korea and Greece,  
19   respectively, Simon and Sanjeev (2007) focused on frozen products and dried fish products  
20   sold in India, and Basti et al. (2006) examined smoked and salted Iranian fish products.  
21   Similarly, González-Rodríguez et al. (2002) only surveyed vacuum-packed cold-smoked  
22   freshwater fish.

23   It was also found surprising that most previous studies dealt with low risk fishery products,  
24   such as fresh fish, frozen products and salted or dried fish products, and hardly a work on the  
25   incidence of *S. aureus* in fishery products having to comply with legal regulations in Spain or

1 other European Union member states was found to be published in the last ten years. As an  
2 exception, a study on vacuum-packed cold-smoked freshwater fish by González-Rodríguez et  
3 al. (2002) reported that 3 packages out of 54 were contaminated with *S. aureus*. In addition,  
4 Norman et al. (2003) reported an incidence of 10% in a particular raw-eaten Italian fishery  
5 product, i.e. strips of cuttlefish, and Alarcón et al. (2006) detected the presence of *S. aureus* in  
6 1 out of 10 ready-to-eat products within a study aimed to establish a RTQ-PCR procedure  
7 suitable for detection and quantification of *S. aureus* in food.

8 Incidence was found to be high in all categories (10-43%), but notable differences were found  
9 among them. Interestingly, the incidence was higher in those categories not covered by  
10 legislation, that is, fresh products, frozen products and salted fish, than in those having to  
11 comply with legal regulations in force at the time the study was conducted, i.e. ready-to-eat  
12 products and ready meals. This result seems to underline the effectiveness of regulations on  
13 the efforts of the industry to ensure food hygiene.

14 Although incidence was lower in fishery products subject to legal regulations, the presence of  
15 *S. aureus* was also detected in a high proportion of ready-to-cook products, smoked fish, non-  
16 frozen surimis, fish roes and other ready-to-eat products. Fishery products of these categories  
17 do not need to be cooked prior to being consumed. Therefore, the risk for the consumer can  
18 become significant if *S. aureus* is above regulatory limits, and food exceeding legal limits  
19 cannot be placed on the market or must be recalled. However, this study has revealed that a  
20 significant proportion of fishery products marketed in Galicia (11.3%) did not comply with  
21 regulatory limits in force.

22 A previous work on cold-smoked fish obtained at retail level in a nearby location showed that  
23 3 out of 54 packages (5.5%) were contaminated with *S. aureus* at levels lower than 4 log  
24 CFU/g (González-Rodríguez et al., 2002), but authors did not report if levels were higher than  
25 regulatory limits (O. 2/8/1991) or laid down in Commission Recommendation 2001/337/EC).

1 In the present study, a higher proportion of smoked fish (18.6%) was found not to comply  
2 with legal regulations.

3 Incidence was highest in fresh products, followed by frozen products. Incidence values  
4 reported for fresh products in previous studies were also high, ranging between 10 and 30%,  
5 with the highest value for those marketed in Northwest Spain (Herrera et al., 2006). These  
6 results were slightly lower to those presented in this work. Incidence was also reported to be  
7 slightly lower in frozen products sold in India, i.e. 17% (Simon and Sanjeev, 2007). In the  
8 present work, samples were homogenized in a lower volume of diluent and the volume of  
9 bacterial suspension spread on agar plates was higher than or equal to those used in all  
10 aforementioned studies. These slight differences in the methodology resulted in a higher limit  
11 of detection, and it could account for at least part of the differences.

12 Conditions such as low-temperature storage, particularly in frozen fish, a low water activity  
13 typical of frozen and salted products or the activity of specific spoilage microorganisms of  
14 fresh fish prevent *S. aureus* to grow and, as a result, enterotoxin production. Also, fresh,  
15 frozen and salted products are commonly cooked prior to being consumed and it should  
16 destroy all or most *S. aureus*. The risk for the consumer is thus lower and no regulatory limits  
17 have been laid down for these fishery products neither in Spanish nor European legislation.  
18 However, an improper storage (temperature abuse) or processing (e.g. long desalting), can  
19 enable SEs to be formed. For instance, desalting at 20°C was found to result in unsafe levels  
20 of *S. aureus* ( $\geq 10^6$  CFU/g) in cod, and possible toxin formation (Pedro et al., 2004). The risk  
21 can increase if the number of microorganisms is low (such as in thawed products), shelf-life is  
22 long (such as in salted products) or the product is consumed raw, undercooked or, in general,  
23 lightly processed. Additionally, staphylococcal enterotoxins (SEs) are highly heat-resistant  
24 and therefore, in many cases, thermal processes cannot be used as a measure to prevent  
25 staphylococcal food poisoning (Balaban and Rasooly, 2000; Cremonesi et al., 2005).

1 Most of the *S. aureus* isolates found in this work carried *se* genes so their incidence in fishery  
2 products marketed in Galicia was high (23.5%). A lower proportion of *S. aureus* *se* positive  
3 had been previously found among isolates from fishery products in other geographical regions  
4 (Normanno et al., 2005; Oh et al., 2007; Simon and Sanjeev, 2007).

5 At present, nine different serological types of SEs (SEA-SEE and SEG-SEJ) have been  
6 proven to have emetic activity (Ortega et al., 2010). Except for two, all *se*-carrying isolates  
7 found in this study carried the *sea* gene, and therefore could produce SEA, but none was  
8 found to be *seb-see* positive. Classical staphylococcal enterotoxins (SEA-SEE) have been  
9 reported to cause 95% of staphylococcal food poisoning. Among them, SEA is the most  
10 common in staphylococcus-related food poisoning (Pinchuk et al., 2010), probably due to a  
11 very high resistance to proteolytic enzymes (Le-Loir et al., 2003). Several studies have  
12 reported that a high proportion of isolates from outbreaks of staphylococcal food poisoning  
13 occurring in South Korea, France, Japan and United Kingdom could produce SEA, either  
14 alone or with another toxin (Cha et al., 2006; Kérouanton et al., 2007; Shimizu et al., 2000;  
15 Wieneke et al., 1993). In contrast, *sea* had been found not to be the most predominant *se* gene  
16 in fishery products in some previous studies (Normanno et al., 2005; Simon and Sanjeev,  
17 2007).

18 Only four of the isolates were multi-*se*-carriers, two harboured the *seg* and *sei* genes and two  
19 others, the *sea*, *sec* and *seh* genes. In contrast, Cha et al. (2006) found that most isolates (ca.  
20 85%) from staphylococcal food poisoning incidents in South Korea were multi-*se*-carriers and  
21 detected *seg-sei* genes in a significant proportion of isolates, either alone (ca. 4%) or along  
22 with other *se* genes (17.5%). Nonetheless, SEG and SEI have been considered to play minor  
23 role in food poisoning (Chen et al., 2004).

24 A dose lower than 1 µg of SE has been reported to make symptoms of staphylococcal food  
25 poisoning to appear within 1-6 h after consumption of contaminated food in an adult healthy

1 individual (Pinchuk et al., 2010; FDA, 2009). This toxin level can be reached when cell  
2 number exceeds  $10^5$  CFU/g of food (Bathia and Zahoor, 2007). As a preventive measure,  
3 legal limits of  $10^2$ - $10^3$  CFU/g had been set for *S. aureus* in different fishery products.  
4 However, in this study, counts of *se*-carrying *S. aureus* exceeded such limits in a significant  
5 number of products and in some of them even by one or two orders of magnitude.

6 *S. aureus* has been reported as the third major causative agent of foodborne illness by fish and  
7 fishery products in the European Union (EFSA, 2009). Moreover, the actual incidence of  
8 staphylococcal food poisoning is known to be much higher than reported (Lawrynowicz-  
9 Paciorek et al., 2007; Smyth et al., 2004). Thus, the notification of staphylococcal  
10 intoxications is not mandatory in many member states of the European Union and most cases  
11 are not reported to healthcare services as they resolve within 24 to 48 h after onset -  
12 hospitalization rate was 19.5% for verified outbreaks caused by *S. aureus* in 2008 (EFSA,  
13 2010)-.However, microbiological criteria laid down in national regulations (O. 2/8/1991 and  
14 RD 3484/2000) have been recently repealed (RD 135/2010) following Commission  
15 Regulation (EC) No 2073/2005 on microbiological criteria for foodstuff. Coagulase positive  
16 staphylococci (mainly *S. aureus*) are thus no longer a microbiological criterion for ready-to-  
17 cook products and most ready-to-eat products. At present, *S. aureus* is set as a process  
18 hygiene criterion only for shelled and shucked products of cooked crustaceans and molluscan  
19 shellfish, with a value for M of  $10^3$  CFU/g. In the present study, 3 out of 12 products (25%)  
20 exceeded this value.

21 The emergence of multi-drug resistant pathogens is recognized as an environmental hazard to  
22 the food supply and human health, as it makes eradication more difficult and incidence to  
23 increase (Livermore, 2000; Popovich et al., 2007; Ribeiro et al., 2007). *S. aureus* has  
24 developed multidrug resistance worldwide, but wide variations in incidence exist regionally  
25 (Gündogân et al., 2006; Normanno et al. 2007; Peles et al., 2007; Pesavento et al., 2007). All

1 isolates found in fishery products marketed in Galicia were resistant to penicillin,  
2 chloramphenicol and ciprofloxacin and most of them were resistant to tetracycline too.  
3 Beleneva (2011) also found a high incidence of ciprofloxacin-resistant *S. aureus* (84.7%) in  
4 fishery products from the Sea of Japan and South China Sea, but the percentage of penicillin-  
5 and tetracycline-resistant strains was lower (47.2% and 27.5%, respectively). Variations in  
6 antibiotic resistance are also the result of other different factors. For instance, Pereira et al.  
7 (2009) isolated a high number of penicillin-resistant *S. aureus* (73%) from meat and dairy  
8 products in a nearby geographical area (North of Portugal), but the number of  
9 chloramphenicol-, ciprofloxacin- and tetracycline-resistant isolates was extremely low (0-  
10 2%). Tetracycline was the only antibiotic on which both resistant- and sensitive-strains were  
11 found. Tetracycline-resistance seemed to enhance the presence of *S. aureus* in fishery  
12 products obtained at retail level in Galicia.

13 Methicillin-resistant *S. aureus* (MRSA) are being increasingly found outside clinical settings  
14 (Popovich et al., 2007; Ribeiro et al., 2007; Stankovic et al., 2007). MRSA have thus been  
15 found in food animals (Lee, 2003) and different foods (Gündoğan et al., 2006; Peles et al.,  
16 2007; Pereira et al., 2009; Pesavento et al., 2007) and also in fishery products recently  
17 (Beleneva, 2011). Although there is currently no evidence that eating food contaminated with  
18 MRSA may lead to an increased risk of humans becoming healthy carriers or infected with  
19 this bacterium (EFSA, 2010), it is important to take some preventive control measures. In the  
20 present study, however, no MRSA was isolated from fishery products and no isolate carried  
21 the *mecA* gene, though intermediate resistance to methicillin was detected in all isolates.

22 The identification of bacterial clones with enhanced virulence or increased ability to spread is  
23 important. RAPD is a fast and cost-effective PCR method for typing and differentiation of  
24 bacterial strains with no prior information of the gene sequence. However, a lack of  
25 standardization and a low reproducibility have been claimed as major drawbacks of RAPD

1 (Deplano et al., 2000; Van-Belkum et al., 1995). Nevertheless, RAPD-PCR binding patterns  
2 were found to be reproducible in this study when DNA from different cultures of a same  
3 isolate was used as a template. A good reproducibility has been also observed in  
4 epidemiological studies using RAPD for typing *S. aureus* isolates (Aras et al., 2012; Fueyo et  
5 al., 2001; Nikbakht et al., 2008; Shehata, 2008). Accordingly, RAPD could be used for initial  
6 screening of isolates in public health epidemiological studies (outbreak and endemic strains),  
7 prior to other complementary typing methods such as pulsed-field gel electrophoresis (PFGE)  
8 and multilocus sequence typing (MLST), which must be used for global epidemiology and  
9 population genetic studies of *S. aureus* (Al-Thawadi et al., 2003; Byun et al., 1997; Deplano  
10 et al., 2006).

11 The use of RAPD to discriminate strains has been also questioned. Morandi et al. (2010) has  
12 recently reported that multilocus variable number tandem repeat analysis (MLVA) is more  
13 powerful than RAPD-PCR for typing of *S. aureus* (discriminatory power of 0.99 and 0.94,  
14 respectively). Nonetheless, the discriminatory power of RAPD was determined by using only  
15 one primer (AP-4). In the present study, the combination of RAPD fingerprints obtained in  
16 separate reactions with three different primers allowed the discriminatory power of the  
17 analysis to be increased (increases of 11.7%, 24.6% and 32.8% for primers S, ERIC-2 and  
18 AP-7, respectively). Other authors have used even a much higher number of primers for strain  
19 differentiation (Byun et al., 1997; Nema et al., 2007). This allows strains to be differentiated  
20 when RAPD patterns are rather similar, i.e. low-yield patterns. However, the number of  
21 polymorphic bands generated by primers S, AP-7 and ERIC-2 was considered to be high  
22 enough to distinguish *S. aureus* strains among isolates found in this study. Nonetheless, it is  
23 not unlikely that the use of a higher number of primers had increased the number of different  
24 strains, but this had been time-consuming.

1 Isolates sharing a same global RAPD fingerprint also showed identical enterotoxin gene and  
2 antibiotic susceptibility patterns, and this supports the thesis that RAPD analysis allowed  
3 bacterial clones to be distinguished. Nema et al. (2007) also found that *S. aureus* isolates with  
4 a same RAPD fingerprint had identical *se* gen patterns and suggested a clonal origin of  
5 isolates.

6 Cluster analyses based on similarity measurements between RAPD patterns found no  
7 relationship between any RAPD pattern and any product category and, though *se*-negative  
8 and *se*-positive strains did not shared RAPD fingerprints, no further relationship was found  
9 between the presence of *se* genes and any RAPD pattern either.

10 The use of RAPD-PCR fingerprinting with several primers to assess the genetic relationship  
11 between *S. aureus* isolated from fishery products marketed in Galicia has therefore exclude a  
12 clonal origin.

13

1   **4. Conclusions**

2   A significant proportion (~25%) of fishery products surveyed from retail sector in Galicia in  
3   2008 and 2009 was found to be contaminated with *S. aureus*, mostly with *se*-carrying strains.

4   About 12% of products did not comply with regulatory limits, and a higher proportion of  
5   products not subject to regulations were contaminated too. These results suggest some effect  
6   of regulations on the efforts of the industry to ensure food hygiene.

7   However, a number of microbiological criteria laid down in national regulations have been  
8   recently repealed and coagulase positive staphylococci (mainly *S. aureus*) are thus no longer a  
9   microbiological criterion for ready-to-cook products and most ready-to-eat products.

10   However, *S. aureus* has been reported as the third major causative agent of foodborne illness  
11   by fish and fish products in the European Union (EFSA, 2009). In addition, the actual  
12   incidence of staphylococcal food poisoning is known to be much higher than reported -the  
13   notification of is not mandatory in many member states of the European Union and most  
14   cases are not reported to healthcare services as they resolve within 24 to 48 h after onset-.

15   A revision of pre-requisite programs and an improvement of hygienic practices in handling  
16   and processing operations from fishing or farming to retail outlet is therefore recommended in  
17   order to ensure the safety of fishery products marketed in Galicia. Nonetheless, at present, *S.*  
18   *aureus* is still under surveillance by a significant part of the industrial sector.

19

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8

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**Table 1.** Nucleotide sequences of primer pairs and predicted sizes of resulting PCR products.

<i>se</i> gene	Primer	Nucleotide sequences (5'→3')	Amplicons Size (bp)	Reference
<i>sea</i>	SEA-3	CCTTGAAACGGTAAAACG	127	Becker et al. (1998)
	SEA-4	TCTAACCTCCCATCAAAAAC		
<i>seb</i>	SEB-1	TCGCATCAAACGTACAAACG	477	
	SEB-4	GCAGGTACTCTATAAGTGCCTGC		
<i>sec</i>	SEC-3	CTCAAGAACTAGACATAAAAGCTAGG	271	
	SEC-4	TCAAAATCGGATTAAACATTATCC		
<i>sed</i>	SED-3	CTAGTTGGTAATATCTCCTTAAACG	319	
	SED-4	TTAATGCTATATCTTATAGGGTAAACATC		
<i>see</i>	SEE-3	CAGTACCTATAGATAAAGTTAAAACAAGC	178	
	SEE-2	TAACTTACCGTGGACCCTTC		
<i>seg</i>	SEG-1	AAGTAGACATTGGCGTTCC	287	Omoe et al. (2002)
	SEG-2	AGAACCATCAAACCTCGTATAGC		
<i>seh</i>	SEH-1	GTCTATATGGAGGTACAACACT	213	
	SEH-2	GACCTTACTTATTCGCTGTC		
<i>sei</i>	SEI-1	GGTGATATTGGTAGGTAAAC	454	
	SEI-2	ATCCATATTCTTGCCTTACCAAG		

**Table 2.** Antibiotic breakpoints used for interpretation of susceptibility tests (S: sensitive; R: resistant).

Antibiotic	<sup>a</sup> CEP	<sup>a</sup> MET	<sup>a</sup> OXA	<sup>b</sup> CIP	<sup>b</sup> CLI	<sup>b</sup> CLO	<sup>b</sup> ERY	<sup>b</sup> GEN	<sup>b</sup> PEN	<sup>b</sup> TET	<sup>b</sup> TRI-SUL	<sup>b</sup> VAN	
<b>Breakpoints (<math>\mu\text{g/ml}</math>)</b>	<b>S <math>\leq</math></b>	8	8	2	1	0.25	8	1	1	0.125	1	2	2
	<b>R &gt;</b>	32	16	4	1	0.50	8	2	1	0.125	2	4	2

CEP: cephalothin; CLI: clindamycin; CLO: chloramphenicol; ERY: erythromycin; GEN: gentamicin; OXA: oxacillin; PEN: penicillin G; TET: tetracycline; VAN: vancomycin; MET: methicillin; CIP: ciprofloxacin; TRI-SUL: trimethoprim-sulfamethoxazole.

<sup>a</sup> Breakpoints from the CLSI (2011).

<sup>b</sup> Breakpoints from the EUCAST (2011).

1    **Legends**

2    **Figure 1.** Incidence (%) of *S. aureus* in fishery products marketed at retail level in Galicia.

3    The number of products surveyed in each category is shown (*n*).

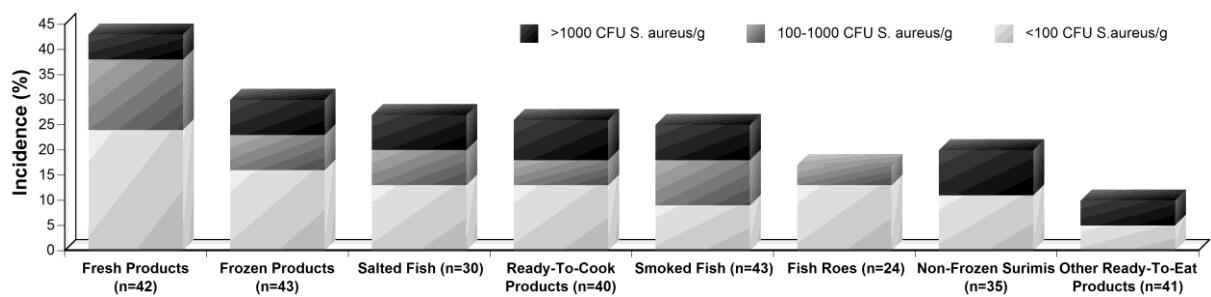
4    **Figure 2A-C.** Agarose gels showing RAPD-PCR fingerprints obtained for *S. aureus* isolates  
5    using primers S (A), AP-7 (B) and ERIC-2 (C). Lane 1: DNA Molecular Weight Marker  
6    (HyperLadder II, 50-2000 bp; Bioline). Pattern number is shown on the top of each  
7    fingerprint.

8    **Figure 3.** Dendrogram from cluster analysis based on the global combination of RAPD-PCR  
9    patterns obtained with all three primers. Combined patterns were assigned a three-digit code  
10   number, with digits corresponding to numbers assigned to patterns obtained with primers S,  
11   AP-7 and ERIC-2, respectively.

12   **Figure 4.** The presence of *se* genes (%) in *S. aureus* isolated from fishery products marketed  
13   at retail level in Galicia. Presence of *se* genes was defined as the number of *se*-carrying *S.*  
14   *aureus*-containing products respect to the number of *S. aureus*-containing products. The  
15   number of fishery products carrying *se* positive *S. aureus* in each category is shown (*n*).

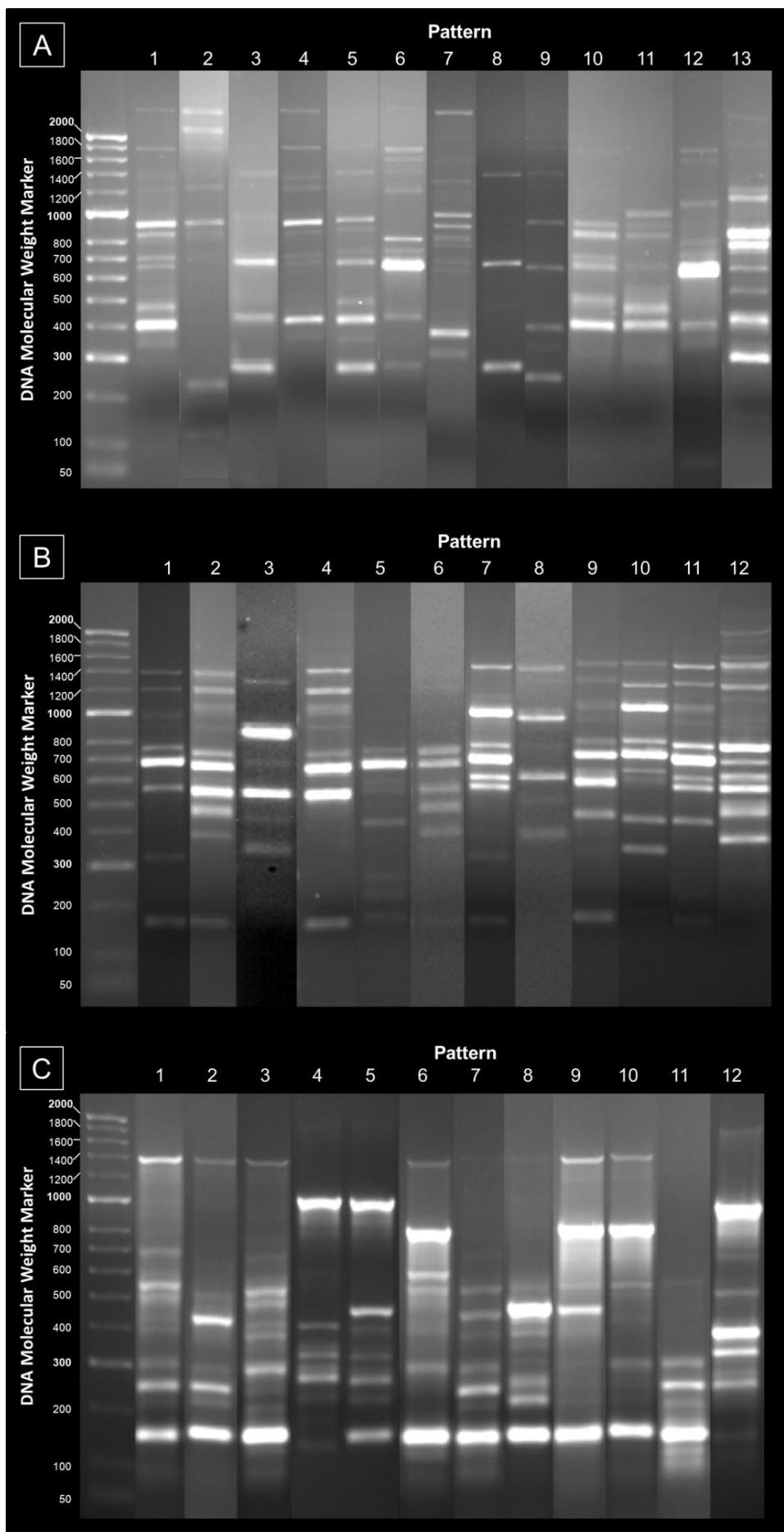
16   **Figure 5.** Tetracycline resistance (%) of *S. aureus* isolated from fishery products marketed at  
17   retail level in Galicia. Resistance was defined as the number of tetracycline-resistant isolates  
18   with respect to the number of isolates. The number of isolates from each category is shown  
19   (*n*).

20

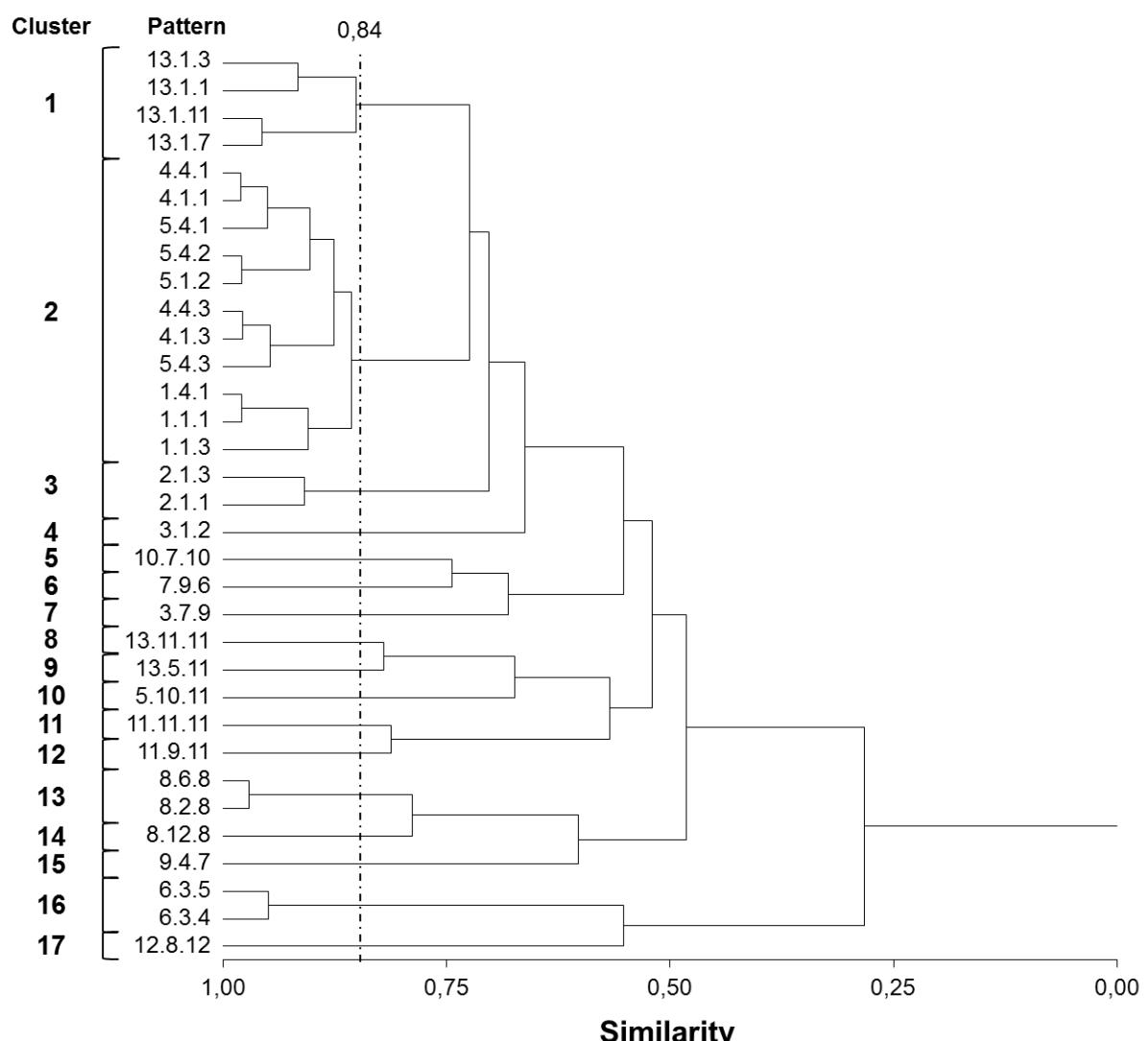


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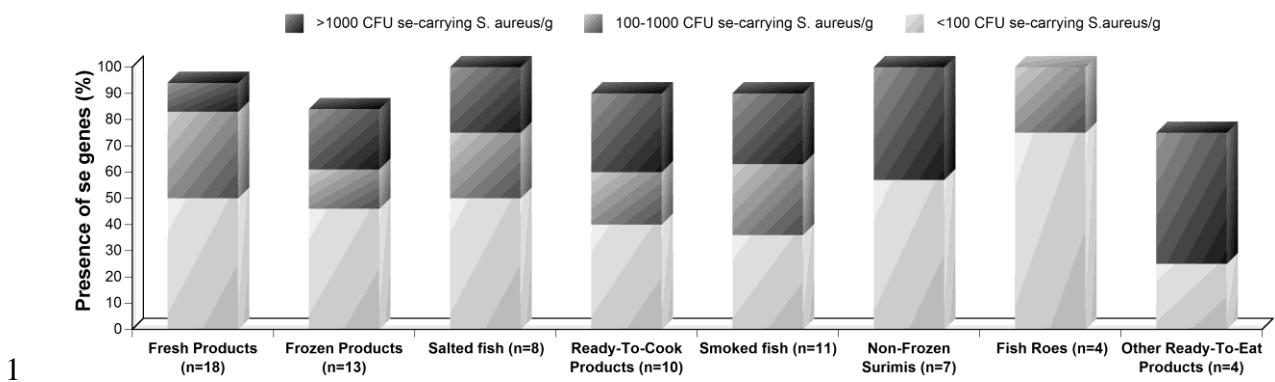


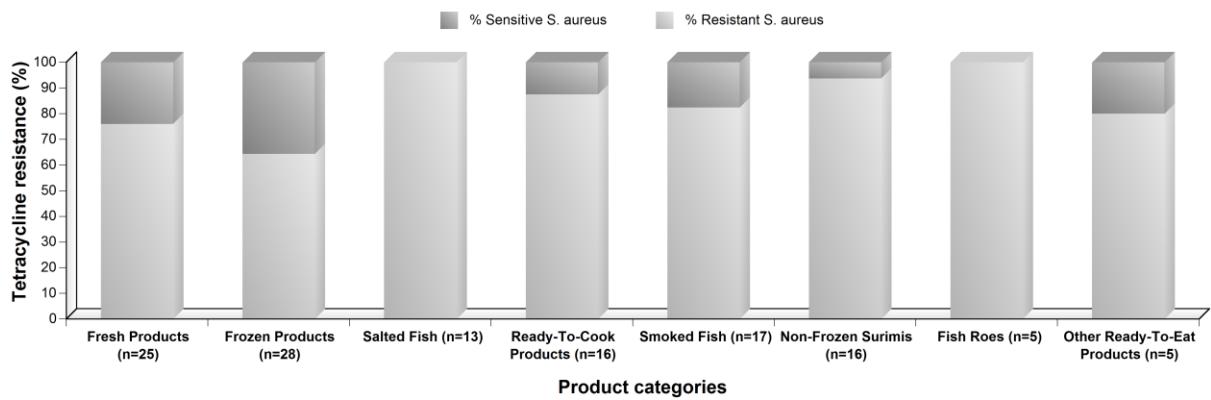
## Primers S. AP-7. ERIC-2



1

2





1