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Impact of food-related environmental factors on the adherence and biofilm formation of natural Staphylococcus aureus isolates

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Introduction

Staphylococcus aureus is a common human pathogen responsible for food-borne intoxications worldwide, caused by the ingestion of food containing staphylococcal heat-stable enterotoxins [26, 28]. The greatest risk of staphylococcal food poisoning is associated with food contaminated with *S. aureus* after the normal microflora has been destroyed or inhibited [5]. In 2009, the European Union witnessed staphylococcal outbreaks which led to a hospitalisation rate of 16.9% [16]. Both food products and food contact surfaces are often contaminated through handling during processing and packaging [14, 43, 44], as *S. aureus* is part of the normal microbiota associated with human skin, throat and nose. Consequently, *S. aureus* has been repeatedly detected in a diverse variety of food, including seafood [22, 32, 36]. One recent study [49] reported a high incidence of *S. aureus* (~25%) in seafood marketed in Spain, which is the largest seafood producer and the second largest consumer in the European Union [17].

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Biofilm is considered as part of the normal life cycle of *S. aureus* in the environment [34], in which planktonic cells present attach to solid surfaces, proliferating and accumulating in multilayer cell clusters embedded in an organic polymer matrix. This structure protects the bacterial community from environmental stresses, from the host immune system and from antibiotic attacks, as opposed to the situation for vulnerable and exposed planktonic cells [9]. This may contribute to the persistence of *S. aureus* in food-processing environments, consequently increasing cross-contamination risks as well as subsequent economic losses due to recalls of contaminated food products. Several studies have shown the attachment of *S. aureus* on work surfaces such as polystyrene, polypropylene, stainless steel and glass, and also in food products [8, 14, 22, 43, 44]. However, changes in surface physicochemical properties and substratum topography, as well as in environmental factors such as osmolarity, nutrient content and temperature may lead to staphylococcal biofilm development and, consequently, influence their persistence on food contact environments [1, 2, 6, 25, 31, 35, 38, 39, 41, 51].

The extracellular matrix of *S. aureus* is mainly composed by poly-β(1,6)-N-acetyl-d-glucosamine (PIA/PNAG), which are synthetized by N-acetylglucosaminyltransferase [10; 18; 30; 33]. This enzyme is induced by the coexpression of *icaA* with *icaD*, products of the chromosomal intercellular adhesion (*ica*) operon carried by most *S. aureus* strains [10; 18; 23; 30]. The expression of the *ica* operon is controlled by the repressor *icaR*, which is regulated by the stress-induced sigma factor B (σ^B) [7] and indirectly by the *rbf* gene [13], among others. These genes are also involved in the resistance of *S. aureus* to various environmental stresses [19; 27; 40].

The present study aimed at investigating the persistence of 26 natural *S. aureus* isolates on polystyrene surfaces, a material frequently used in the food industry, through the evaluation of their physicochemical, adhesion and biofilm-forming properties under different environmental stress conditions found during processing, packaging and storage of food products. Moreover, the variability of the expression of genes implicated in the regulation of biofilm formation between three strains selected during the screening was also investigated under different stress conditions.

Materials and Methods

Bacterial strains and growth conditions

Twenty six *S. aureus* isolates from seafood marketed in Galicia (Northwest Spain) were investigated. They were previously identified as *S. aureus* by specific biochemical (coagulase, DNAse and mannitol fermentation) and genetic tests (*23s* rDNA) and characterized by RAPD-PCR [49]. These isolates carried *sea* (n=22), *sea-c-h* (n=2) or *seg-i* (n=2) genes, whose expression produce enterotoxins. *S. aureus* ATCC 6538 (a known biofilm former) and *S. aureus* ATCC 43300 (MRSA strain), provided by the Spanish Type Culture Collection (Valencia), were used as reference strains. Stock cultures were maintained in 20% glycerol at -80°C. All strains were thawed and subcultured in tryptic soy broth (TSB, Oxoid, UK) for 24 h at 37°C, 200 rpm prior to use.

Evaluation of bacterial cell surface physicochemical properties

Microbial Adhesion to Solvents (MATS) was used as a method to determine the hydrophobic character of the cell surface of *S. aureus* strains and their Lewis acid-base properties [4]. This method is based on the comparison between microbial cell surface affinity to a monopolar solvent and an apolar solvent, which both exhibit similar Lifshitz-van der Waals surface tension components. Chloroform (an electron-acceptor solvent), hexadecane (nonpolar solvent), ethyl acetate (an electron-donor solvent) and decane (nonpolar solvent) were used of the highest purity grade (Sigma-Aldrich, USA). Experimentally, overnight bacterial cultures were washed twice in phosphate buffer (7.6 $g \cdot l^{-1}$NaCl, 0.2 $g \cdot l^{-1}$KCl, 0.245 $g \cdot l^{-1}$ Na_2HPO_4 and 0.71 $g \cdot l^{-1}$ K_2HPO_4; Merck, Inc.) and resuspended to a final OD_{400nm} of 0.8 (~10^8 $CFU \cdot ml^{-1}$). Individual bacterial suspensions (2.4 ml) were first mixed with 0.4 ml of the respective solvent and then manually shaken for 10 s prior to vortexing for 50 s. The mixture was allowed to stand for 15 min to ensure complete separation of phases. 1 ml from the aqueous phase was removed and the final OD_{400nm} measured. The percentage of cells residing in the solvent was calculated by:

$$\%Adherence = \frac{(OD_i - OD_f)}{OD_i} \times 100$$

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where (OD_i) was the optical density of the bacterial suspension before mixing with the solvent and (OD_f) the absorbance after mixing and phase separation. Each measurement was performed in triplicate and the experiment was performed twice using independent bacterial cultures.

Measurement of the adherence ability to polystyrene at different ionic strength conditions

The ability of *S. aureus* strains to adhere to polystyrene was evaluated in terms of biomass using the crystal violet method described by Giaouris et al. [20], but with some modifications. Overnight cultures were washed twice and resuspended to a final OD_{600nm} of 0.8 in 150 mM NaCl or 1.5 mM NaCl. 200 µl of each sample was added in a flat-bottomed 96-well microtiter plate with Nunclon Surface (Nunc, Denmark) and then incubated for 4 h at 25ºC. After measuring the OD_{600nm}, the microplates were washed three times with peptone water (Oxoid, UK), using an automatic microplate washer (Wellwash AC, Thermo Electron Corporation, Inc.), and air-dried for 2 h. Wells were then stained for 15 min using 150 µl of 0.5% (w/v) Crystal Violet (CV) (Merck, Inc.) followed by three rinsing steps with distilled water. The microplates were air-dried for 15 min and the bound CV was extracted with 150 µl of 33% (v/v) Glacial Acetic Acid (Merck, Inc.) for 30 min at room temperature. 100 µl of the mixture was diluted in a new microplate with 100 µl of 33% Glacial Acetic Acid prior to read the OD_{562nm}. Each measurement was performed in triplicate and the experiment was repeated twice using independent bacterial cultures.

Quantification of biofilm formation on polystyrene under different environmental conditions

The biofilm-forming ability of *S. aureus* strains on polystyrene microtiter plates was also investigated in terms of biomass, using an optimized protocol based on previously described methods [37, 41, 45]. Each well was added with 100 µl of growth medium and 100 µl of an overnight bacterial culture diluted 1:100 in TSB. Negative control wells contained TSB only. Biofilm formation was evaluated after 24 and 48 h in TSB with or without 5% glucose, 5% NaCl, 5% glucose + 5% NaCl, 0.1 mM $MgCl_2$ or 1 mM $MgCl_2$ (Merck, Inc.) at 25 and 37°C. After measuring the OD_{600nm}, the microplates were washed three times with peptone water using the automatic microplate washer and air-dried for 2 h. The microplates were then stained with 150 µl of 0.5% (w/v) CV for 15 min followed by three rinsing steps with distilled water. After air-dried for 15 min, the bound CV was extracted with 150 µl of 33% (v/v) Glacial Acetic Acid for 30 min. The mixture added to a new microplate was then diluted 1:1 in 33% Glacial Acetic Acid prior to read the OD_{562nm}. Each measurement was performed in triplicate and the experiment was repeated twice using independent bacterial cultures.

Transcriptional analysis

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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

1 To assess the expression levels of the genes reported in Table 1, RNA was extracted from St.1.07, St.1.14 and
2 St.1.29 grown in TSB with or without 5% glucose, 5% NaCl or 5% glucose + 5% NaCl. An overnight culture
3 was diluted 1:100 in each medium and cultivated at 37ºC with 200 rpm of agitation until an OD_{600}~0.5. After
4 incubation, two volumes of bacterial culture were diluted in four volumes of RNAprotect Bacteria Reagent
5 (Qiagen, Hilden, Germany). The mixture was vortexed for 15 s, incubated for 5 min at room temperature and
6 centrifuged (5000 × g) for 10 min at room temperature. The supernatant was discarded and 200 µL of a mixture
7 containing TE buffer, 40 mg·ml^{-1} lysozyme and 1 mg·ml-1 lysostaphin (Sigma, USA) was added for enzymatic
8 lysis of bacteria. RNA was isolated using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany), following
9 the manufacturer's instructions and including a DNase treatment. The concentration and purity of total RNA
10 were analyzed using a NanoDrop, ND-1000 spectrophotometer (NanoDrop Technologies, Inc.).
11 Reverse transcription of the RNA isolated was carried out using random primers, as previously described [41]
12 with slight modifications. A reaction mixture (13 µl) with 300 ng RNA, 100 ng Random Primers and 10 mM of
13 each dNTP (Invitrogen) was denatured at 65°C for 5 min, incubated on ice immediately for at least 1 min and
14 centrifuged briefly. A mixture (6 µl) of 5x first strand buffer, 0.1 M DTT and 200 U Superscript III reverse
15 transcriptase (Invitrogen) was then added to the reaction. The samples were incubated at 25°C for 5 min, heated
16 at 50°C for 45 min and immediately incubated at 70°C for 15 min to inactivate the reaction. A brief
17 centrifugation between each step was done. Six reverse transcriptase reactions were made for each biological
18 replicate of RNA, of which three were without enzyme as negative controls.
19 Quantitative real-time PCR (qRT-PCR) was performed in an Abi Prism 7900 HT Sequence Detection System
20 (Applied Biosystems, Inc.). The PCR mixture contained 1× TaqMan Buffer A, 5 mM $MgCl_2$, 0.2 mM of dATP,
21 dCTP and dGTP, 0.4 mM dUTP, 0.2 µM primer, 0.1 µM probe, 0.1 U AmpErase uracil N-glycosylase, 1.25 U
22 Ampli-Taq Gold DNA Polymerase (Applied Biosystems, Roche, Inc.), 10 ng of cDNA and dH_2O ultrapure
23 DNAse and RNAse free (Gibco, Invitrogen Corporation) up to a final volume of 25 µl. Primers and Taqman®
24 probes were designed previously by Rode et al. [41]. Reaction mixtures were subjected to an initial cycle of
25 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min.
26 C_T values were estimated on SDS 2.2 software (Applied Biosystems, Inc.). The difference between C_T of the
27 reference gene *16S* and C_T of other gene analyzed (ΔC_T) were calculated to see possible changes in gene
28 expression. One unit change represents a log of 2-fold change.
29 *Statistical analysis*

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Results from the analytical determinations were statistically treated with the software package IBM SPSS 19.0. They were averaged and the standard error of the mean was calculated. Data of the adhesion and biofilm formation assays were normalized and expressed as OD_{562nm}/OD_{600nm}, due to the variation in total growth at 25ºC and 37ºC and to have a clearer view of biofilm formation for the conditions where growth was limited, as Rode et al. [41] proposed. Significance of the data was determined using a one way ANOVA and the homogeneity of variances was examined by a post-hoc least significant difference (LSD) test. Otherwise, a Dunnett´s T3 test was performed. An independent-samples T test was also done to compare strains in pairs. Bivariate correlations were analyzed using the Pearson correlation coefficient. Significance was expressed at the 95% confidence level ($P<0.05$) or greater. Principal Components Analysis (PCA) was performed to group the 28 *S. aureus* strains by their similar physicochemical, adhesion and biofilm formation properties showed on polystyrene. Varimax normalization method with Kaiser was used to build the rotated component matrix.

Results

Cell surface hydrophobicity and electron donor/acceptor character

The physicochemical surface properties of the 28 *S. aureus* strains were studied to estimate their potential for adhesion and subsequent biofilm formation on surfaces. Affinities of the strains to different polar and apolar solvents are presented in Fig. 1. Considerable variations in the percentage of adhesion to decane between *S. aureus* tested strains reveal the degree of diversity in their hydrophobic character. Affinity to decane ranged from 22.32% to 74.82%. However, affinity to hexadecane were less variable ranging from 56.40% to 84.14%, revealing a moderate hydrophobic character for the majority of *S. aureus* tested strains. High percentage of adhesion to chloroform was observed for all tested strains (ranging between 74.37% and 95.75%), which in all cases were higher than that to hexadecane. This also reveals the diversity in electron donor (Lewis base) properties among tested *S. aureus*, highlighting the strain St.1.19 with the highest electron donor character. *S. aureus* tested strains generally expressed non electron acceptor (Lewis acid) properties, as seen by the higher affinity to decane compared to ethyl acetate with values below 19.75%.

Adherence ability of S. aureus to polystyrene surfaces

Initial adhesion to polystyrene surfaces of the 28 *S. aureus* strains was quantified in terms of biomass at two different ionic strengths (1.5 mM and 150 mM NaCl) to evaluate their electrostatic interactions. The results showed that initial adhesion to polystyrene was positively correlated (r=0.577, P<0.01) with ionic strengths presented in the suspension. Thus, initial adhesion to polystyrene was reduced at lower ionic strength conditions compared to high ionic conditions, except for strains St.1.08 and St.1.21 (Fig. 2). Moreover, the variability of

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adhesive properties to polystyrene among *S. aureus* strains at low ionic strength medium may also be an indication of the diversity in cell wall electronegativity among the tested *S. aureus* strains, as previously described [20]. The strains St.1.08 and St.1.09 showed the most remarkable adherence ability under low and high ionic strength conditions, respectively.

Biofilm formation on polystyrene surfaces under different environmental conditions

The ability of the 28 *S. aureus* strains to develop biofilms on polystyrene surfaces under different conditions of temperature (25ºC and 37ºC), osmolarity and nutrient content (TSB with or without 5% glucose, 5% NaCl, 5% glucose + 5% NaCl, 0.1 mM $MgCl_2$ and 1 mM $MgCl_2$) was investigated after 24 and 48 h to understand the effects of environmental factors in staphylococcal biofilm formation. These two temperatures were selected by their relevance to the food industry and hospitals (25ºC) and in infectious disease (37ºC). To compensate for variations in cell mass at stationary phase at the two different temperatures, the biofilm formation values were expressed as OD_{562nm}/OD_{600nm}. Significant differences ($P<0.05$) between strains for each treatment and viceversa were observed, as indicated by the different letters showed in Fig. 3.

Effect of incubation temperature. Biofilm formation in a medium without nutrient addition (TSB only) was positively correlated ($r=0.386$, $P<0.01$) with the temperature of incubation. Thus, incubation at 37ºC increased biofilm-forming ability for the majority of tested isolates (84%), compared to incubation at 25ºC. *S. aureus* St.1.22 and St.1.11 showed the highest biofilm formation at 37ºC, while St.1.31 was able to form biofilms with high cell densities at 25ºC (Fig. 3a).

Biofilm formation was also positively correlated ($P<0.01$) with incubation temperature when TSB was added with 5% glucose ($r=0.522$), 5% glucose + 5% NaCl ($r=0.637$), 0.1 mM $MgCl_2$ ($r=0.487$) or 1 mM $MgCl_2$ ($r=0.405$), but addition of 5% NaCl generated a negative correlation ($r=-0.418$, $P<0.01$). In fact, 78.5% of the strains showed a higher biofilm formation in TSB with 5% NaCl when they were incubated at 25ºC than at 37ºC.

Effect of glucose and NaCl addition. Addition of 5% glucose to TSB generally led to enhanced staphylococcal biofilm formation (Fig. 3b), as shown its positive correlation ($P<0.01$) with biofilm formation under all tested conditions (Table 2). However, these increases on biofilm development with the addition of glucose were affected by incubation temperatures. The highest increases in biofilm formation with the addition of 5% glucose were produced in the first 24 h at 37ºC and after 48 h at 25ºC, with 3-fold and 2-fold increases respectively. In the presence of 5% glucose, isolates St.1.01, St.1.02, St.1.04 and St.1.08 expressed a 4-fold biofilm increase after 24 h at 37ºC, while isolates St.1.05 and St.1.06 showed 5-fold increases after 48 h at 25ºC.

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The effect of NaCl on biofilm formation was markedly affected by incubation temperatures. Thus, a negative correlation ($P<0.01$) at 37ºC between biofilm formation and the addition of 5% NaCl was observed (Table 2). In fact, 75% of tested isolates expressed lower biofilm formation in environments with supplemented salt than those grown in the absence of salt (Fig. 3c). Nevertheless, a positive correlation ($P<0.01$) was reported at 25ºC for the first 24 h between NaCl addition and biofilm formation, slightly improving the production of biofilm by most isolates (75%). Under similar conditions, isolates St.1.02, St.1.21 and St.1.29 grown in the presence of 5% NaCl showed a remarkable 2-fold increase in biofilm formation compared to those grown in the absence of salt. These isolates were isolated from a Paella (containing mussels and squids), frozen shelled prawns and a Panga fillet respectively, three seafood products with high amounts of NaCl (>100 mg per 100 g of product) [46]. No significant correlation was observed after 48 h at 25ºC between biofilm formation and the addition of NaCl. Comparing with individual effects, no synergy was observed between the addition of glucose and NaCl (Fig. 3d). Moreover, no significant correlations were observed between biofilm formation and the addition of both nutrients, except a negative correlation ($P<0.01$) reported when the strains were incubated for 24 h at 37ºC. The addition of 5% glucose + 5% NaCl therefore slightly increased the biofilm formation compared to growth in the absence of glucose and NaCl in 64.3% of all tested isolates after 48 h growth for both 25ºC and 37ºC. Two-fold biofilm increases were observed in non-supplemented TSB for isolates St.1.07, St.1.12 and St.1.28 grown at 25ºC, and isolates St.1.03, St.1.05, St.1.06, St.1.08 and St.1.14 grown at 37ºC.

Effect of $MgCl_2$ addition. Generally, addition of 0.1 mM $MgCl_2$ did not significantly affect biofilm formation compared to growth in the absence of $MgCl_2$ (Fig. 3e). No correlation was observed between the addition of 0.1 mM $MgCl_2$ and biofilm formation, except a positive correlation ($P<0.05$) for growth at 37ºC after 48 h (Table 2). In fact, 57.1% of the strains increased significantly their biofilm formation with the addition of $MgCl_2$ under these conditions, highlighting St.1.05, St.1.07, St.1.14, St.1.20 and St.1.31 with a 3-fold biofilm increase. Otherwise, the increment of the $MgCl_2$ concentration from 0.1 to 1 mM did not induce an increase on the biofilm formation (Fig. 3f). Consequently, only 21.4% isolates showed on average a 2-fold increase with the addition of 1 mM $MgCl_2$ after 48 h at 37ºC, highlighting St.1.01 and St.1.03 isolated from smoked swordfish, a seafood with high magnesium levels (57 mg per 100 g of product) [46]. Biofilm formation was positively correlated ($P<0.05$) with the addition of 1 mM $MgCl_2$ after 48 h at 37ºC, but no significant correlations were observed under the other conditions tested (Table 2).

Multivariate analysis of the physicochemical, adhesion and biofilm-forming properties of the 28 *S. aureus* strains

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The variables (n=30) defined during adhesion and biofilm formation assays as well as two additional variables (the type of seafoods from which isolates were sampled and the type of processing used during their production) were used to perform a Principal Components Analysis (PCA) for the 28 *S. aureus* strains. However, the two principal components (PC) obtained only accounted for 33% of total variance. The selection of the most significant parameters (n=8) indicated in the rotated component matrix for each PC allowed increase up to 79.3% the total variance accounted (Table 3). PC1 and PC2 accounted individually a variance of 55.7% and 23.6%, respectively. PC1 was positively correlated (P<0.01) with biofilm formation in TSB with 5% glucose at 25ºC and in TSB added with or without 5% glucose, 5% glucose + 5% NaCl or 1 mM $MgCl_2$ at 37ºC. PC1 was also correlated with type of product (r=0.444, P<0.05) and processing (r=0.625, P<0.01). Meanwhile, PC2 was positively correlated (P<0.01) with biofilm formation in TSB with 5% NaCl or 5% glucose + 5% NaCl at 25ºC. *S. aureus* isolates were located in a scatter plot based on the results from both PC obtained (Fig. 4). They were distributed in four groups, each one corresponding to a defined quadrant. Considerable variations in the ability to develop biofilms on polystyrene were showed by the isolates under environmental conditions selected. Five isolates were distributed in the first quadrant (delimited by a solid line), which showed a biofilm formation ability significantly influenced by the addition of 5% NaCl alone or together with 5% glucose at 25ºC. Both the biofilm former reference strain ATCC 6538 as well as the two isolates carrying *sea*, *sec* and *seh* genes (St.1.07 and St.1.24) tested in this study were located in this quadrant. However, the five strains had a different origin: ATCC 6538 were isolated from a human lesion, St.1.07 and St.1.28 from fresh fish and St.1.20 and St.1.24 from precooked products. The second quadrant (delimited by dots) included six isolates which biofilm development on polystyrene was highly influenced by the environmental conditions selected, highlighting St.1.04 and St.1.12. However, they were isolated from seafood with a different processing: St.1.01, St.1.06 and St.1.12 were isolated from smoked fish, St.1.02 and St.1.05 from precooked products and St.1.04 from a salted product. The third quadrant (delimited by broken lines) clustered the highest number of strains (n=10), including the antibiotic resistant strain ATCC 43300 and the two strains carriers of *seg* and *sei* genes (St.1.16 and St.1.19) of this study. Biofilm formation of these isolates was not significantly affected by the environmental conditions selected. Given that most of them were isolated from frozen (5) and fresh (2) products, other conditions such as cold temperatures could be the environmental limiting factor during biofilm formation of these isolates. Moreover, two strains (St.1.13 and St.1.15) of this group were isolated from shellfish growth by aquaculture, where the application of antibiotics is widely used. Finally, the fourth quadrant (delimited by dots inserted between broken lines) grouped seven strains which biofilm formation was mainly influenced by the addition to TSB of 5%

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glucose at 37ºC. They were isolated from precooked (St.1.10, St.1.11 and St.1.14), smoked (St.1.22) and salted (St.1.23) products, and two from products made with squids (St.1.09 and St.1.30).

From these results, *S. aureus* St.1.07, St.1.14 and St.1.29 strains were selected for their characteristic biofilm-forming ability under food-related environmental stresses tested to investigate the expression of different genes involved in biofilm formation.

Gene expression in relation to biofilm formation

The genes *icaA*, *rbf* and σ^B are reported to be involved in the regulation of biofilm formation. Their statistical significant (P<0.05) changes in expression were investigated under different biofilm promoting growth conditions (TSB with 5% glucose, 5% NaCl or 5% glucose + 5% NaCl) and compared with expression in TSB by reverse transcriptase real-time PCR for the three selected strains. All the genes were highly expressed in TSB (C_T≤30), with significant (P<0.05) differences between the strains. Thus, St.1.14 showed the highest expression of *icaA* (C_T=26.9) and *rbf* (C_T=28.4) genes, whereas gene σ^B was highly expressed by St.1.07 (C_T=24.6).

Each strain showed a different expression pattern of the analysed genes under the different growth conditions tested. The most variable expression was observed in *icaA* gene (Fig. 5A). An additive effect on *icaA* expression was seen in St.1.07 when both NaCl and glucose were added, whereas *icaA* expression in St.1.29 was down-regulated in high NaCl conditions (without glucose additions) and up-regulated by the presence of glucose in the medium. In contrast, *icaA* expression in St.1.14 was highly affected by the presence of NaCl, while an up-regulation was observed upon glucose addition. Otherwise, the genes *rbf* and σ^B were also highly expressed by the three strains selected (Fig. 5B-C). In St.1.07, expression of these genes was up-regulated by NaCl with a dominant down-regulating effect of glucose. For strain St.1.14, expression of σ^B was increased when glucose, NaCl or both were added, whereas expression of *rbf* was up-regulated by the presence of glucose in the medium. Finally, an additive effect on *rbf* expression was also seen in St.1.29 when both NaCl and glucose were added, whereas expression of σ^B was up-regulated by glucose addition.

Discussion

The present study showed considerable variations between the adhesion and biofilm formation properties of 26 natural *S. aureus* isolates from seafoods on polystyrene surfaces under different food-related environmental stress conditions. This surface is frequently used in the food industry, above all in the packaging of products, and its bacterial colonization may cause food-spoilage, consequently increasing risk for the consumer health as well as subsequent economic losses due to recalls of contaminated food products.

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Bacterial adhesion to surfaces is directly correlated with cell surface hydrophobicity [35, 42]. According to our results, all *S. aureus* strains expressed moderate hydrophobicity, suggesting a lower initial adhesion to hydrophobic polystyrene compared to hydrophilic surfaces such as glass. Mafu et al. [29] also reported a moderate hydrophobicity and a low tendency to attach to polystyrene in *S. aureus*, but a single strain was used.

The electrostatic interactions between the tested *S. aureus* strains and polystyrene surface showed a significantly (P<0.01) higher adhesion when the ionic strength conditions were increased from 1.5 mM NaCl to 150 mM NaCl, except for strains St.1.08 and St.1.21. As previously reported [20, 21], adhesion at high ionic conditions was probably caused by the attenuation of repulsive electrostatic interactions between the highly negatively charged bacteria and the negatively charged polystyrene surface. The initial adhesion of *S. aureus* to polystyrene could therefore be enhanced in situations involving the use of seawater during seafood-processing, consequently increasing the risk of biofilm formation and cross-contamination. Therefore, the use of fresh water as a mean to reduce the attachment of negatively charged bacteria to polystyrene should be considered. Moreover, obtained results showed that initial adhesion was dependent on both tested strain and ionic strength conditions. A high variability in initial adhesion to polystyrene among *S. aureus* strains was observed for both ionic conditions, hence suggesting possible differences in cell wall electronegativity, as described by Giaouris et al. [20] in *Lactococcus lactis*. To our knowledge, this is the first time that such variability of surface physicochemical properties is described for natural *S. aureus* strains from fisheries. Therefore, these findings provide important information for the development of novel surfaces and control strategies against the adhesion of natural *S. aureus* during processing, packaging and storage of food products, especially in fisheries.

Principal Components Analysis also showed a considerable variability in biofilm formation between the 26 *S. aureus* strains tested under relevant environmental conditions of temperature, osmolarity and nutrient content found during seafood production. Thus, isolates had generally higher biofilm production at 37ºC as expected, although four strains (St.1.14, St.1.16, St.1.24 and St.1.31) showed a significantly higher biofilm development during the first 48 h at 25ºC. Pagedar et al. [35] also reported a higher cell count of *S. aureus* growth in TSB at 25ºC than at 37ºC after 48 h, but these biofilms were formed on stainless steel surfaces.

Meanwhile, the presence of glucose increased biofilm formation of all tested *S. aureus*, although significant differences between isolates were observed. This nutrient is considered a limiting factor of biofilm formation due to its requirement during the production of the extracellular matrix components [2]. Therefore, our results are in totally agreement with those obtained by Rode et al. [41], considering that the presence of glucose promotes biofilm formation in *S. aureus*. In fisheries, glucose is an additive frequently used to reduce the water activity of

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products, above all in surimis and smoked fish. Data obtained in this study showed that the presence of glucose significantly influenced biofilm formation of most isolates (70%) from surimis and smoked fish, as shown their distribution in the PCA score plot. Thus, the presence of glucose in these products could potentially increase the contamination by *S. aureus*, involving a serious risk for the health of consumers and probable economic losses.

Another important environmental factor is the amount of NaCl present on food-processing surfaces, which could be increased by the presence of seawater and seafood wastes generated during seafood production. Different authors showed that NaCl could promote bacterial aggregation and enhanced the stability of biofilms in polystyrene [31, 41]. However, the addition of NaCl generally decreased the biofilm formation of tested *S. aureus* strains at 37ºC, whereas it was improved at 25ºC. Xu et al. [51] reported that the number of adhered cells of *S. aureus* ATCC 12600 in polystyrene was higher in a medium without NaCl for the first 48 h at 37ºC. A possibility proposed by Lim et al. [27] could be the repression of biofilm formation either directly or through overexpression of *rbf* gen with concentrations of 5% NaCl approximately. However, a rather average expression of *rbf* gen was observed in this study during transcriptional analysis by qRT-PCR of *S. aureus* St.1.07, St.1.14 and St.1.29 -isolates selected by their characteristic biofilm-forming properties for PCA- when they were growth in TSB added with 5% NaCl. Rachid et al. [40] described an osmotic stress resistance and biofilm formation induced by σ^B, but a lower expression of σ^B was reported in *S. aureus* St.1.29, which had a remarkable biofilm formation in the presence of 5% NaCl compared to those grown in the absence of salt. Therefore, these results indicate a great variability of regulatory responses against osmolarity stress conditions during the development of staphylococcal biofilms. Further investigations (e.g. using knock-out mutants) should be done in the future to deepen this study. Results of such studies could lead to new biofilm control strategies on food contact surfaces.

Several authors also indicated the influence of $MgCl_2$ in the adhesion to food contact surfaces of *Staphylococcus* spp. [1, 3, 15, 38]. In fisheries, both seawater and seafood wastes are an important source of magnesium. However, biofilm formation of *S. aureus* isolates tested in this study generally was not affected by the presence of $MgCl_2$, although rather favoured after 48 h at 37ºC. These results are in accordance as those previously reported, suggesting that $MgCl_2$ are implicated in biofilm stabilization at optimal growth conditions.

The results obtained in this study hence supported that environmental conditions found in the food industry affected the adhesion and biofilm formation in *S. aureus*. Different regulatory pathways are involved in biofilm development of *S. aureus* highlighting the *ica* operon, which is associated in the regulation of extracellular matrix synthesis [10]. Several authors reported that the addition of glucose, NaCl or both together promote biofilm formation by inducing the *ica* operon in *S. aureus* [31, 41]. In this study, all the tested strains carried

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icaA and *icaD* (results not shown). Moreover, an increase in *icaA* expression with the addition of glucose was also observed during transcriptional analysis by qRT-PCR of the selected *S. aureus* isolates St.1.07, St.1.14 and St.1.29. However, although *icaA* expression remained high, biofilm formation was lowered when both glucose and NaCl were added, suggesting that other *ica*-independent pathways are implicated as proposed previously different authors [18, 24]. Other internal factors supposedly involved in the initial adhesion to surfaces and host molecules and in the intercellular adhesion are the biofilm-associated proteins or Bap [12]. However, none of the natural *S. aureus* isolates from seafoods carried *bap* gen. These results are in accordance with Vautor et al. [48], which concluded that the prevalence of this gene among *S. aureus* isolates should be very low. In fact, the *bap* gene has only been identified in a small proportion of *S. aureus* strains originating from bovine mastitis [11].

Conclusions

According to results obtained in the present study, natural *S. aureus* seems to show a high ability to adhere and form biofilms on polystyrene surfaces. Food-contact surfaces made of this material can thus be a hazardous reservoir for *S. aureus* in the food industry and, therefore, an important source of food contamination unless appropriate food safety procedures are applied.

Our results also support that staphylococcal biofilm formation is influenced by environmental conditions relevant for the food industry such as temperature, osmolarity, nutrients content and cell surface properties. In fact, considerable variations in biofilm-forming ability were observed between the different strains tested under these environmental conditions. Therefore, the prevalence of *S. aureus* isolates on food contact surfaces may be linked to their ability to adapt to the environmental stresses present during food production.

These findings are relevant for food safety and may be of importance when choosing the safest environmental conditions and material during processing, packaging and storage of seafood products. The maintenance of thermal conditions that avoid or reduce the bacterial growth in food products, the use of low-adherent materials in food-processing facilities as well as the application of proper cleaning and disinfection procedures to food contact surfaces are essentials to ensure food safety.

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Table 1 Primers and Taqman probes used in the study, with the function and the nucleotide sequences.

Gene	Name	Sequence 5′ → 3′	Accesion no.	Reference
16S	16S-Pr	CGTAGGTGGCAAGCGTTATCCGGA	NC002951	[5]
	16S-F	CCAGCAGCCGCGGTAAT		
	16S-R	CGCGCTTTACGCCCAATA		
icaA	icaA-Pr	TGGATGTTGGTTCCAGAAACATTGGGAG	BX571857	[47]
	icaA-F	TGAACCGCTTGCCATGTG		
	icaA-R	CACGCGTTGCTTCCAAAGA		
rbf	rbf-Pr	TGGATGTTGGTTCCAGAAACATTGGGAG	BX571857	[27]
	rbf-F	TTAGAAGGAATCTTTAAAACCTTATTGAATAA		
	rbf-R	TTGTGAATTTTTCTTCTTCGGACA		
σ^B	sigB-Pr	AGAAGTGTTAGAAGCAATGGAAATGGGACAAAGTTATAAT	Y09929	[50]
	sigB-F	ATAGCTGATCGATTAGAAGTCTCAGAAG		
	sigB-R	TCAATGGAATGATCAACACTTAACG		

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Table 2 Correlations between the biofilm formation and nutrient content expressed as *r* values. An *r* value of zero indicates no correlation, whereas a value of 1 or -1 indicates a perfect positive or negative correlation.

Nutrient added	Incubation condition			
	25ºC 24 h	25ºC 48 h	37ºC 24 h	37ºC 48 h
5% glucose	0.478[b]	0.588[b]	0.733[b]	0.470[b]
5% NaCl	0.499[b]	0.082	-0.521[b]	-0.439[b]
5% glucose + 5% NaCl	-0.031	-0.040	-0.356[b]	0.133
0.1 mM $MgCl_2$	0.148	0.033	0.149	0.176[a]
1 mM $MgCl_2$	0.139	0.049	0.068	0.161[a]

[a] $P<0.05$; [b] $P<0.01$

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Table 3 Component score coefficients matrix obtained from the PCA for the eight relevant parameters selected, which account for 79.3% of the total variance.

Indicator	Condition	PC 1	PC 2
TSB	37ºC 24h	0.843	-0.254
TSB + 5% glucose	25ºC 48h	0.754	0.122
TSB + 5% glucose	37ºC 24h	0.921	-0.055
TSB + 5% glucose	37ºC 48h	0.928	-0.003
TSB + 5% NaCl	25ºC 48h	0.030	0.938
TSB + 5% glucose + 5% NaCl	25ºC 48h	-0.072	0.949
TSB + 5% glucose + 5% NaCl	37ºC 48h	0.885	-0.127
1 mM $MgCl_2$	37ºC 24h	0.825	0.108

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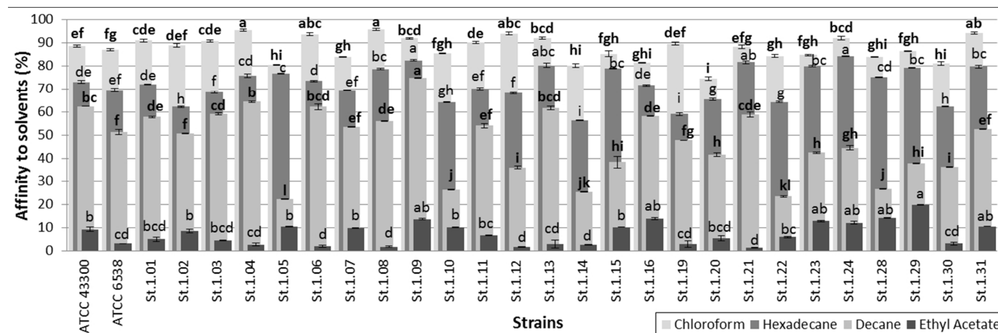

Fig. 1 Affinity of S. aureus strains (n=28) to the solvents chloroform, hexadecane, decane and ethyl acetate. Mean and SD values: three replicates of each sample. Different letters on the top of each column show significant differences (P<0.05) in affinity to each solvent between the strains tested.
234x78mm (150 x 150 DPI)

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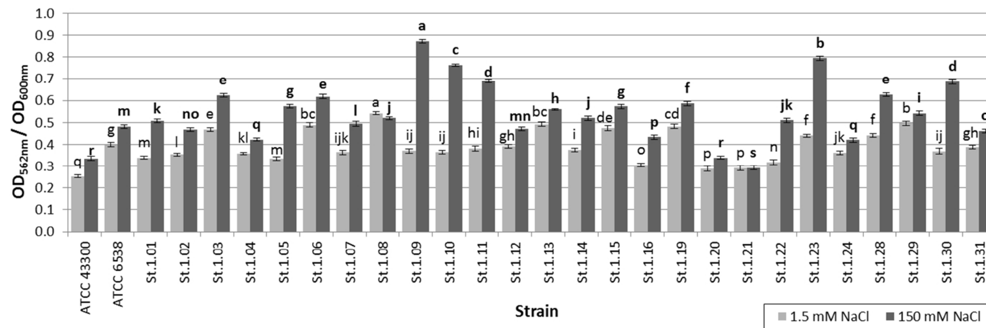

Fig. 2 Initial adhesion to polystyrene surfaces of S. aureus strains (n=28) under different ionic strength conditions (NaCl 1.5 mM and 150 mM). Adhesion ability of each strain was expressed in terms of biofilm biomass after 4 h at 25ºC. Mean and SD values: three replicates of each sample. Significant differences (P<0.05) between the adherence ability of strains at each condition were indicated by different letters on the top of each column.
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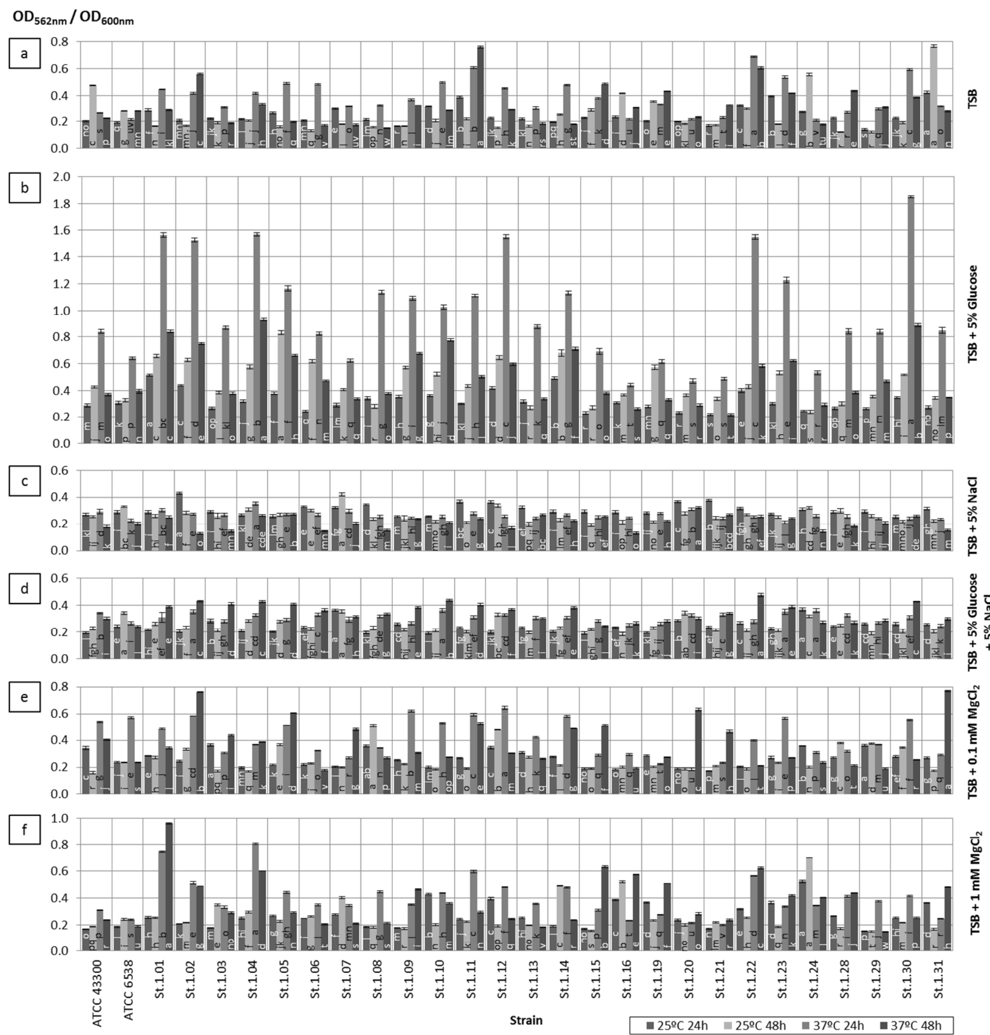

Fig. 3 Biofilm formation of S. aureus strains (n=28) on polystyrene in TSB only (a) or added with 5% glucose (b), 5% NaCl (c), 5% glucose + 5% NaCl (d), 0.1 mM MgCl2 (e) or 1 mM MgCl2 (f). Mean and SD values: nine replicates of each sample. Different letters on each column indicate significant differences (P<0.05) in biofilm formation between strains for each condition tested.
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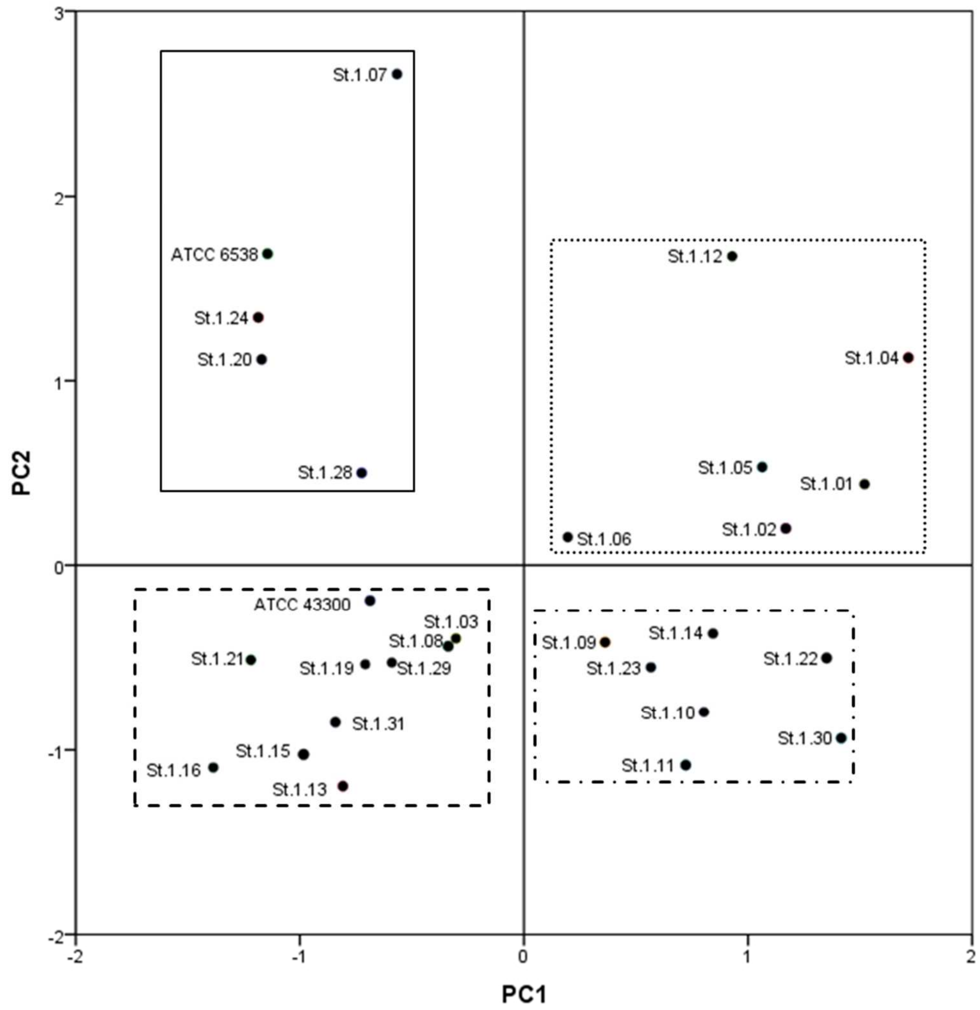

Fig. 4 PCA score plot of the S. aureus strains (n=28) for first two components. PC1: impact on biofilm formation of glucose at 25ºC and glucose, glucose + NaCl and MgCl2 at 37ºC. PC2: impact of NaCl and glucose + NaCl on biofilm formation at 25ºC. First quadrant delimited by a solid line; second quadrant delimited by dots; third quadrant delimited by broken lines; fourth quadrant delimited by dots inserted between broken lines.
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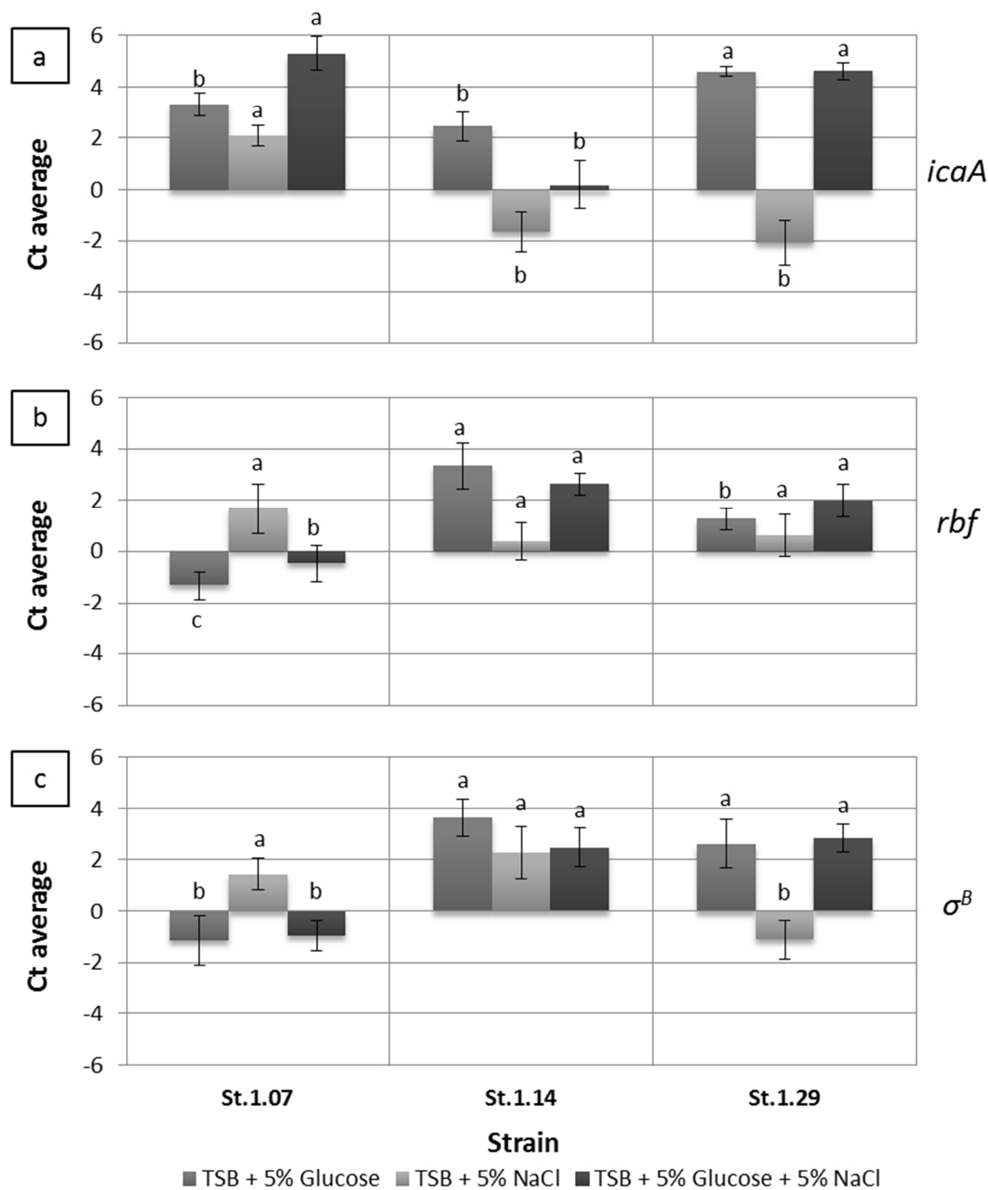

Fig. 5 ΔCt for the expression of genes icaA (a), rbf (b) and σB (c) in three S. aureus strains (St.1.07, St.1.14 and St.1.29) under different conditions compared to expression in TSB. Different letters on the top of each column show significant differences (P<0.05) in the expression of these genes at each condition tested between the selected strains.
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