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Genotype and enterotoxigenicity of *Staphylococcus epidermidis* isolate from ready to eat meat products

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Abstract

We have previously shown that potentially pathogenic isolates of *Staphylococcus epidermidis* occur at high incidence in ready-to-eat food. Now, within 164 samples of ready-to-eat meat products we identified 32 *S. epidermidis* isolates. In 8 isolates we detected the genes encoding for staphylococcal enterotoxins, but in 7 *S. epidermidis* isolates these genes were not stable over passages. One isolate designated 4S was shown to stably harbour *sec* and *sel* genes.In the genome sequence of *S. epidermidis* 4S we identified 21,426-bp region flanked by direct-repeats, encompassing *sec* and *sel* genes, corresponding to the previously described composite staphylococcal pathogenicity island (SePI) in *S. epidermidis* FRI909. Alignment of *S. epidermidis* 4S and *S. epidermidis* FRI909 SePIs revealed 6 nucleotide mismatches located in 5 of the total of 29 ORFs. Genomic location of *S. epidermidis* 4S SePI was the same as in FRI909. *S. epidermidis* 4S is a single locus variant of ST561, being genetically different from FRI909. SEC_{epi} was secreted by *S. epidermidis* 4S to BHI broth ranging from 14 to almost 36 μg/mL, to milk ranging from 6-9 ng/mL, to beef meat juice from 2-3 μg/mL and to pork meat juice from 1-2 μg/mL after 24 and 48 hours of cultivation, respectively. We provide the first evidence that *S. epidermidis* occurring in food bears an element encoding an orthologue to *S. aureus* SEC, and that SEC_{epi} can be produced in microbial broth, milk and meat juices. Regarding that only enterotoxins produced by *S. aureus* are officially tracked in food in EU, the ability to produce enterotoxin by *S. epidermidis* pose real risk for food safety.

Keywords

Staphylococcus epidermidis; staphylococcal pathogenicity island; staphylococcal enterotoxin; SEC; food safety

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1. Introduction

Genus *Staphylococcus* comprises 52 species and 28 subspecies (http://www.bacterio.net) of gram-positive, non-motile bacteria that frequently occur as commensal colonizers of the mucocutaneous membranes of the warm-blooded animals and human (Becker et al., 2014; Fitzgerald and Penadés, 2008; Wertheim et al., 2005). With regard to the ability to coagulate rabbit plasma staphylococci fall into coagulase-positive (CPS) and coagulase-negative (CNS) groups, however this division does not reflect heterogeneity in the pathogenicity and habitat preferences within taxon. For decades, most of research was focused on coagulase-positive *Staphylococcus aureus;* hence, its virulence factors, population structure, pathogen-host interactions, and ability to cause life-threatening infections remain characterised to the greatest extent (Bergdoll, 1989; Lowy, 1998). *S. aureus* is also well known for its ability to evoke food poisoning due to the secretion of heat stable enterotoxins that may express superantigenic activity (Hennekinne et al., 2012; Le Loir et al., 2003). Several CNS species (e.g. *S. carnosus*, *S. xylosus,* and *S. equorum*) are widely applied in food industry, exerting positive impact on fermentation processes and sensory characteristics of meat products (Nilsen and Rødbotten, 2007). Recent research highlighted the need of studies on involvement of CNS in human and animal disease. Enterotoxigenic CNS strains were already isolated from cases of human clinical infections (Ataee et al., 2011; de Cuhna et al., 2007; Vasconcelos et al., 2011), and foodstuffs (Even et al., 2010; Marín et al., 1992; Rall et al., 2010; Rodríguez et al., 1996; Zell et al., 2008). CNS isolates endowed with enterotoxigenic properties were also isolated from either healthy or diseased animals (Adesiyun and Usman, 1983; Park et al., 2011; Unal and Cinar, 2012; Valle et al., 1990). To date, the only well characterized enterotoxigenic CNS is *S. epidermidis* FRI909 strain isolated in the 1960's from a human clinical case (Madhusoodanan et al., 2011). FRI909 was shown to harbour *sec* and *sel* genes on an element similar to *S. aureus* pathogenicity island (hence designated SePI), and to express SEC and SEL. As it was first described by Park et al. (2011), some enterotoxin genes seem to occur in CNS in unstable form (Piette and Verschraegen, 2009). The significance of these genes for food safety, and public health remains unknown (Podkowik et al., 2013).

Among CNS *Staphylococcus epidermidis* has gained the greatest attention, so far. Involvement of *S. epidermidis* in serious hospital infections especially in device-associated cases has been proven (Otto, 2004, 2009; Ziebuhr, 2001).Our previous studies have shown high incidence of *S. epidermidis* in ready-to-eat (RTE) meat products, and have confirmed significant prevalence of potentially pathogenic isolates among them (Podkowik et al., 2012a, 2012b). Therefore we aimed to describe incidence and characteristics of enterotoxigenic *S. epidermidis* isolates derived from RTE meat products.

2. Materials and methods

2.1. Isolation and identification of *S. epidermidis*

One hundred and sixty four samples of ready-to-eat porcine, bovine and chicken meat products were screened for presence of staphylococci. The samples were taken during a thirteen-month period from seven randomly selected supermarkets in Wrocław, Poland. Staphylococci from food samples were cultured on Giolitti-Cantoni enrichment broth

(Thermo Fisher Scientific Inc., Waltham, MA, USA) and then subcultured onto Baird-Parker agar (Thermo Fisher Scientific Inc.). One isolate per product was taken for further analyses. The *Staphylococcus epidermidis* isolates were identified by API Staph ID 32 (bioMerieux, Marcy l'Etoile, France), and additional tests for catalase, clumping factor and coagulase were done. Simultaneously, *tuf* (Martineau et al., 2001) and 16S rDNA genes (primers from htpp://rdna4.ridom.de) were partially sequenced.

2.2. Preparation of bacterial DNA

Two millilitres of bacterial cell suspension from an overnight culture grown in brain-heart infusion (BHI) broth (Thermo Fisher Scientific Inc.) were centrifuged for 5 min at 12,000 × g and suspended in 100 μL of 100 mM Tris-HCl buffer, pH 7.4, containing 10 μg of lysostaphin (A&A Biotechnology, Gdynia, Poland). After 30-minute incubation at 37 °C, 10 μL of 10% SDS was added and the sample was incubated for another 30 min at 37 °C. Two hundred μL of 5 M guanidine hydrochloride were added and the sample was mixed and incubated at room temperature for 10 min. The DNA was extracted by phenol and chloroform, precipitated with ethanol, and dissolved in water.

2.3. Detection of enterotoxin genes

Detection of SEs genes (sea-see, seg, seh, sei, selj, sek, sem, seo, tst1, sel, sen, sep, seq, ser, selu) was performed with the use of primers and cycling conditions described by Park et al. (2011). The only modification was setting separate reactions for detection of every SE gene. All the amplicons in the size close to expected were sequenced. Sequencing was performed with the BigDye Terminator ready-reaction cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). *S. aureus* reference strains FRI913, A900322, FRI1151m, and CCM5757 served as PCR controls (Table 1).

2.4. Assessment of enterotoxin genes stability in *S. epidermidis* isolates

Enterotoxigenic *S. epidermidis* isolates were cultured on Columbia Agar with 5% sheep blood (BTL, Łódź, Poland) at 37 °C. The bacteria were subcultured every 24 hours. The entire procedure comprised 10 consecutive passages. A wire loop of bacterial cells (several randomly selected colonies) was taken from each passage for further DNA isolation and analysis of enterotoxin gene content. During the cultivation colony morphology was assessed focussing on potential colony heterogeneity. The stability of *S. epidermidis* enterotoxin genes was also assessed following freezing at −80 °C in 30% glycerol. In addition, enterotoxigenic *S. epidermidis* 4S isolate was subjected to 10 consecutive passages in liquid microbiological medium. In précis, 100 μL of 24-hour *S. epidermidis* planktonic culture was transferred everyday into 5 mL of fresh BHI broth and grown at 37 °C with agitation (230 rpm). Each day 500 μL of 24-hour bacterial culture was harvested and centrifuged, and then the pellet was used for DNA isolation. PCR for respective enterotoxin genes was performed on the DNA from each bacterial passage. The same procedure was performed in altered temperature or shaking velocity (20 °C and 80 rpm, respectively).

2.5. Genome sequencing and analysis of *S. epidermidis* 4S isolate

An indexed, paired-end sequencing library was prepared for *S. epidermidis* 4S isolate using a Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA, USA) and cleaned with AMPure XP beads (Agencourt Bioscience, Beverly, MA, USA). The library was sequenced on an IlluminaMiSeq instrument using a 600 cycle Reagent Kit v3 (Illumina), which yielded 2.47 million paired-end reads for this isolate. CLC Genomics Workbench v7 was used to assemble the reads *de novo* after trimming and filtering for base quality of Q13 (equivalent to base error probability of ≤0.05), number of ambiguities ≤2, and length ≥15 bp. The assembly resulted in 321 contigs ranging in size from 500 bp to 70,462 bp with an N50 of 14,093 bp. Multilocus sequence type (ST) was determined from the genome sequence by extracting the appropriate gene fragments and comparing with the sequences deposited in the international MLST database (sepidermidis.mlst.net). *S. epidermidis* 4S contigs were also aligned against *S. epidermidis* FRI909 SePI sequence (WGS accession number AENR00000000). The sequence gaps between contigs comprising SePI were filled by Sanger dideoxy sequencing, and the resulting SePI sequence was submitted to GenBank and given accession number KT845956.

2.6. Cloning, expression, and purification of rSEC from *S. epidermidis* 4S isolate

The region encoding mature SEC was PCR-amplified from *S. epidermidis* 4S DNA using the Prime STAR HS DNA Polymerase (Takara Bio Inc., Otsu, Shiga, Japan) according to the protocol: 98°C for 2 min; followed by 30 cycles at 98 °C for 10 s; 55 °C for 10 s; 72 °C for 7 s. The forward cloning primer CATGCCATGGGAGAGAGTCAACCAGACC and the reverse cloning primer CGGCTCGAGTCCATTCTTTGTTGTAAG carried the restriction sites for NcoI and XhoI. The product was purified from agarose gel, digested with XhoI and NcoI (Thermo Fisher Scientific Inc.), ligated into the pET-22b plasmid vector (Merck, Kenilworth, NJ, USA)., and introduced using calcium chloride into NovaBlue *Escherichia coli* cells (Merck). The plasmid was purified using NucleoSpin Plasmid (Macherey-Nagel, Düren, Germany) and sequenced. The plasmid containing the intact sequence of the respective region of *sec* was transformed into *E. coli* Rosetta cells (Merck). Expression was performed using the IPTG induction protocol. Briefly, 500mL of freshly inoculated bacterial culture in LB medium was incubated in 37°C, at 230rpm, until $OD_{600}$of 0.7, then IPTG (Sigma-Aldrich, St. Louis, MO, USA) was added to a final concentration of 0.5mM.After 3h of incubation bacterial cells were harvested by centrifugation for 10 min at 5,000 × g in 4 °C. Purification of recombinant SEC (rSEC) was performed on His-Select cobalt affinity gel (Sigma-Aldrich), with on-column refolding. Briefly, the bacterial pellet was lysed in 20 mM Tris-Cl (pH 8.0), containing 50 mM NaH_2PO_4, 150 mM NaCl, 8 M urea, and 5% glycerol. The lysate was centrifuged for 45 min at 16,000 × g, and the supernatant was applied on a column pre-equilibrated with the lysis buffer. The column was washed with lysis buffer, followed by washes with 20 mM Tris-Cl (pH 8.0), 50 mM NaH_2PO_4, 150 mM NaCl, and 5% glycerol containing decreasing concentrations of urea, from 6 M to 0 M. The protein was eluted with 250 mM imidazole in 20 mM Tris-Cl (pH 8.0), 50 mM NaH_2PO_4, 150 mM NaCl, and 5% glycerol. Protein concentration was measured using the Bradford reagent (Sigma-Aldrich). The purity of rSEC preparation was checked with sodium dodecyl sulfate– polyacrylamide gel electrophoresis (SDS-PAGE).

2.7. Culture of *S. epidermidis* 4S in BHI broth, milk and meat juices

Frozen stock culture of *S. epidermidis* 4S was resuscitated by plating on brain heart infusion (BHI) agar supplemented with 1% yeast extract (YE) and incubated at 37 °C overnight. Single colony was transferred to 5 mL of BHI broth with 1%YE in test tube for 18 hours (37 °C, 230 rpm). One hundred microliters of overnight culture was inoculated into fresh BHI broth with 1% YE and grown in the same conditions and incubated for 2 hours. Next, cells pelleted from 1 mL of culture were washed twice with phosphate-buffered saline (PBS) to remove residual media by centrifugation at 12,000 × g for 5 min. The cells were then suspended in 1 mL of PBS. We used sterilized liquid milk (UHT 0.0% fat milk, Mlekpol, Grajewo, Poland), and meat juices obtained from pork and beef meats according to Rantsiou et al. (2012). All used media were confirmed to be bacteriologically negative and determined to be SEC-free based on the ELISA described below. BHI broth with 1% YE and milk inoculated with PBS-washed cells was suspended in 1 mL of PBS to give an optical density at 600 nm (OD_{600}) of 0.02. Cultures were conducted at 37 °C with agitation (230 rpm). Samples for SEC detection were collected after 24 hours of growth, 1.5 mL of each medium was harvested, centrifuged for 5 min at 12,000 × g and supernatant stored at −20 °C until analysed.

2.8. Growth curve determination in *S. epidermidis* 4S

Growth curve was determined for *S. epidermidis* 4S isolate cultivated in BHI broth supplemented with 1% of yeast extract and milk, beef and pork meat juices at 37°C with agitation (230rpm). Cell counts at seven time points were assessed by seeding consecutive 10-fold dilutions of cultures on BHI-agar.

2.9. RNA extraction and Real-time PCR

Bacterial pellets were suspended in 70 μL 100 mM Tris-HCl (pH 7.4), containing 28 U/mL of lysostaphin and incubated for 15 min at 37 °C. RNA was extracted with TRI Reagent® (Sigma-Aldrich) according to manufacturer's instructions. RNA was dissolved in 50 μL of water and quantified by measuring A_{260} and A_{280}. One μg of RNA was treated with RNAse-free DNase I (Sigma-Aldrich) in order to eliminate residual genomic DNA. cDNA was synthesized using random hexamers and SuperScript III® (Life Technologies, Carlsbad, CA, USA) following manufacturer's instructions. Primers used in real-time PCR, SECfor: 5'-CTTGTATGTATGGAGGAATAACAA-3' and SECrev: 5'-TGCAGGCATCATATCATACCA-3' were taken from Monday et al. (1999). Primers for *rpoB*gene, used for normalization of cDNA, were as follows: Rpofor 5'-CTACAAAACCAATTCCGTATCG-3' and Rporev: 5'-TTAATTGTTGAGGTGTGATAGAC-3'. Real-time quantitative PCR was carried out in iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), using iQ™ Sybr® Green Supermix (Bio-Rad). The reaction mixture contained 1 μL of template cDNA, 0.5 μM of each primer, 10 μL of iQSybr Green Supermix and water up to 20 μL. The reaction protocol was: 95 °C, 30 s; 35 repeats of 95 °C, 10 s, 55 °C, 15 s, and 72 °C, 15 s. Specificity of PCR was evaluated by melt curve analysis in a temperature range from 90 to 65 °C performed for each reaction. Residual DNA contamination was checked in each RNA sample by running no-RT controls. PCR efficiencies for each primer pair were determined

on genomic *S. aureus* DNA from respective reference strains by running serial 5-fold dilutions of the template. Determined efficiencies were taken into account when calculating relative transcript levels according to Pfaffl (2001). Two or three independent cultures were performed for each strain.

2.10. Western blotting

The protein concentrations in culture supernatants were measured using the Bradford reagent (Sigma-Aldrich), and 5μg of protein was loaded per well in SDS-PAGE. Western immunoblots were probed with rabbit anti-SEC antibodies (Acris, Herford, Germany). To detect the primary antibody, a conjugate of protein A–horseradish peroxidase (Thermo Fisher Scientific Inc.) was used. The blots were developed using the ECL Lumi-Light substrate (Roche, Basel, Switzerland), according to the manufacturer's instructions.

2.11. Sandwich ELISA

Culture supernatants from reference *S. aureus* strains and *S. epidermidis* 4S were pre-incubated with 20% normal rabbit serum, in order to bind protein A, and diluted 5 times in PBS containing 0.01% Tween-20. ELISA was performed according to the protocol described by Freed et al. (1982), with modifications. Rabbit polyclonal anti-SEC antisera were from Acris. Instead of coupling of the horseradish peroxidase (HRP) to the antibody, the antibody was biotinylated with biotin N-hydroxysuccinimide ester (Sigma-Aldrich). Biotinylated antibody was detected with conjugate of HRP-streptavidin (Sigma-Aldrich). 3.3′, 5.5′-Tetramethylbenzidine (Sigma-Aldrich) was used as a substrate for HRP. The specificity of the ELISA was established using culture supernatants of *S. aureus* reference strains FRI913, A900322, FRI1151m, CCM5757 as controls for enterotoxins SEA, SEB, SEC, SED, SEE, SEG, SEI, SElJ, SEK, SEL, SEM, SEN, SEO, SEP, and SER. The concentration of the enterotoxin in samples was measured with $rSEC_{epi}$ from *S. epidermidis* 4S as a standard, using a 4-parameter logistic curve fit. Data analysis was carried out using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

3.1. Incidence of *S. epidermidis* in ready-to-eat meat products

In 164 samples of ready-to-eat porcine, bovine and chicken meat products obtained from seven randomly selected supermarkets in Wrocław, Poland 32 isolates *S. epidermidis* were identified.

3.2. Detection and stability of enterotoxin genes in *S. epidermidis*

In 8 of 32 studied *S. epidermidis* isolates enterotoxin genes were detected. The *seh* gene was detected in 2 isolates and *seq* in 4 isolates. Two isolates harboured more than one enterotoxin gene, i.e., in one of them the *sec* together with *sel* was detected, and in another one *sen*, *seq* together with *selu* genes were found.

The eight *S. epidermidis* isolates positive for enterotoxin genes were subjected to sequential passages in order to assess SEs genes stability. In 7 *S. epidermidis* isolates a gradual loss of PCR signal for enterotoxin genes was observed, and the signal was completely lost after 4-5

passages on solid medium. This phenomenon comprised all of the detected enterotoxin genes. No difference in colony morphology was observed. Both colony types were tested for the presence of enterotoxin genes using colony-PCR. Additionally, the species affiliation of analysed colonies was corroborated aiming to exclude culture contamination. No contamination by additional species was detected in these analyses. Resuscitation of 30% glycerol stock cultures stored in −80°C revealed loss of PCR signal for enterotoxin genes in all the 7 *S. epidermidis* isolates (bacterial stocks were resuscitated by plating on BHI agar supplemented with 1% YE, and incubated at 37 °C overnight). Reexamination of these 7 *S. epidermidis* isolates from dozen or so days old colonies on solid medium, obtained during primary isolation, also yielded negative results for enterotoxin genes.

Only one isolate of 8 analysed, namely the *S. epidermidis* 4S isolate was found to possess stable enterotoxin *sec* and *sel* genes, with no loss of PCR signal throughout all 10 passages on solid media. This enabled us to perform liquid media passages. Similarly, no loss of PCR signal for enterotoxin genes was observed in this experiment. Storing of stock cultures in 30% glycerol in −80 °C did not influence *S. epidermidis* 4S enterotoxin gene content.

3.3. Genotyping of *S. epidermidis* 4S isolate

Sequence analysis of the 7 loci used for MLST of *S. epidermidis* yielded a previously unknown allele pattern: *arcC49*, *aroE3*, *gtr9*, *mutS5*, a new allele at *pyrR*, *tpiA4*, and *yqiL4*. The new *pyrR* allele resulted in a new sequence type (ST) for *S. epidermidis* 4S that was a single locus variant of ST561.

Previously, the 437 STs in the international MLST database for *S. epidermidis* were clustered into 6 genetic clusters (GCs) using Bayesian methods. An updated analysis of 578 STs shows that ST561 is assigned to GC4. In addition, a subset of 7 SNPs that can rapidly assign isolates to GCs (Tolo and Robinson, manuscript in preparation) places both ST561 and *S. epidermidis* 4S into GC4 with >95% confidence.

3.4. Sequence analysis of *S. epidermidis* 4S genomic island

In the genome sequence of *S. epidermidis* 4S we identified a region comprising 21,426 bp (GenBank Accession number GenBank KT845956), flanked by direct-repeat sequences of 73 bp (DR2) at the 5'end and 15 bp (DR1) and the 3' end. This genomic island encompassed two superantigen genes, i.e., *sec* and *sel,* and corresponds to the previously described composite staphylococcal pathogenicity island (SePI) in *S. epidermidis*. Alignment of *S. epidermidis* 4S and *S. epidermidis* FRI909 SePIs revealed 6 nucleotide mismatches located in 5 of the total of 29 ORFs. Five of them resulted in amino acid substitution (Table 2).Analysis of sequences located downstream and upstream of DRs defining the ends of SePI revealed that in *S. epidermidis* 4S SePI is inserted in the same region as in FRI909.

S. epidermidis 4S *sec* gene was analysed for homology to known *sec* gene sequences. This search revealed its identity to *sec* gene from *S. epidermidis* FRI909 strain. Comparison of translated coding sequence of *S. epidermidis* SEC_{epi} indicated that it is most related to *S. aureus* SEC_3, having 3 amino-acid substitutions in signal peptide and 9 in mature enterotoxin and most distant from SEC_{ovine}, having 8 amino-acid substitutions in signal peptide and 18 in mature enterotoxin (Table 3).

3.5. Expression of *sec* gene in *S. epidermidis* 4S

qRT-PCR was performed aiming to determine growth phase-dependent profile of SEC_{epi} expression. Results were compared with SEC expression profile in *S. aureus* FRI137 strain, in which *sec* gene is known to remain under control of the *agr* quorum sensing system. As expected SEC_{epi} expression increased towards early stationary phase of growth, reflecting the SEC expression pattern observed in FRI137 (Figure 1).

Using Western blotting SEC_{epi} was detected in culture supernatants from *S. epidermidis* 4S. Its expression profile at 3 hour, 6 hour, and 24 hour of cultivation has been compared to SEC produced by *S. aureus* reference strains FRI137 and FRI913(SEC_1 and SEC_3, respectively). In all tested strains SEC was detectable at 3 hour of culture, corresponding to early exponential phase of growth (OD_{600} 0.4- 0.5), and SEC concentration increased with time (Figure 2).

Supernatants from 24 and 48-hour cultures of *S. epidermidis* 4S, FRI137 and FRI913 reference *S. aureus* strains, and wild, food sampled *S. aureus* isolate designated 175 grown in BHI broth, milk, beef and pork meat juices were tested with ELISA for SEC production. *S. aureus* FRI137 (0.1-2 μg/mL) and FRI913 (85-332 ng/mL) strains were shown to secrete to milk several dozen-fold more SEC than *S. epidermidis* 4S (6-9 ng/mL) and *S. aureus* 175 (1-2 ng/mL) did. On the other hand, BHI (14-36 μg/mL) and beef meat juice (2-3 μg/mL) production of SEC exhibited by *S. epidermidis* 4S exceeded SEC amounts elaborated by FRI137 (0.3-30 μg/mL in BHI and 9-15 ng/mL in beef juice), FRI913 (4-12 μg/mL in BHI and 0.5-2 μg/mL in beef juice), and *S. aureus* 175 (236-371 ng/mL in BHI and 112-601 ng/mL in beef juice). *S. epidermidis* 4S secreted more SEC to pork meat juice (1-2 μg/mL) than *S. aureus* FRI137 (27-43 ng/mL) and 175 (133-263 ng/mL), and less than *S. aureus* FRI913 strain (3-6 μg/mL) (Table 4). Analysis of *S. epidermidis* 4S growth in milk indicated it can reach high cell numbers (8.9 log CFU/mL at 24 hour), if compared to tested *S. aureus* strains, but it did not result in high production of SEC by *S. epidermidis* 4S. In turn, despite low cell numbers of *S. epidermidis* 4S in beef meat juice, reaching 6.7 log CFU/mL at 24 hour it could produce the highest amount of SEC (Table 4).

4. Discussion

According to the EC Regulation 1441/2007 on microbiological criteria for foodstuffs, selected food products are being examined for the presence of SEA-SEE. The underlying condition for routine food control is the presence of *S. aureus* exceeding 10^5 CFU/g (http://eur-lex.europa.eu). For this, the food safety cannot be guaranteed when enterotoxins are produced by staphylococcal species other than *S. aureus*. So far, data concerning *S. epidermidis* counts in food products remains obscure. Most studies focus rather on CNS species distribution in food specimens than on their counts. In the study performed by da Cuhna et al., (2006) *S. epidermidis* counts were assessed in bakery goods, milk and sandwiches. Bacteria numbers oscillated from 1.3×10^3 CFU/g in bakery products, and 3.2×10^4 CFU/mL in milk, to 7.2×10^4 CFU/g in ready to eat sandwiches. In these analyses *S. epidermidis* was the most predominant species, accounting for 40% of all CNS, followed by *S. xylosus* (20%), *S. warneri* (20%), *S. saccharolyticus* (15%), and *S. hominis* (5%). It is cumbersome to establish clear relation of staphylococcal loads and food safety risk

associated with enterotoxin production. Complexity of the problem can be illustrated using data from analyzes of milk-associated *S. aureus* isolates. Surveys on bulk milk samples in Hungary and Switzerland indicated low levels of enterotoxigenic *S. aureus* in milk ranging from 10^1 CFU/mL to 10^3 CFU/mL (Peles et al., 2007; Stephan et al., 2002). Moreover, since production of some enterotoxins starts at relatively high bacterial counts some authors argue for low risk associated with SEC-production by *S. aureus* in milk (Hunt et al., 2014). However, some data indicate low starting *S. aureus* counts may easily multiply in favorable conditions reaching densities posing risk of SE secretion. Analysis of large SFP outbreak in Brazil due to the cheese and unpasteurized milk showed that *S. aureus* starting from 10^3 CFU/g were able to produce SEA, SEB, and SEC (Simeao do Carmo et al., 2007). In Norway a SEH-producing *S. aureus* found at 10^3 CFU/mL in product containing raw milk was able to secrete SEH at amounts reaching 0.3μg/mL (Jorgensen et al., 2005).

The role of enterotoxigenic coagulase-negative staphylococci as food safety hazard remains a matter of debate. Moreover, some data on enterotoxin production by CNS seem to be controversial (Podkowik et al., 2013).

Results of this study provide the first evidence of the stable *sec* gene in *S. epidermidis* isolate derived from food. We also confirm SEC_{epi} transcriptional and translational expression in both laboratory medium and food matrix. To date, the only CNS identified to carry stable *sec* gene was *S. epidermidis* FRI909, a strain recovered from human source (Madhusoodanan et al., 2011). Although the data are still scarce, it seems that *S. epidermidis* harbouring stable enterotoxin genes are not frequent in food products or from human infections. Within 32 *S. epidermidis* cultures isolated by us from 164 food samples only one stably harboured *sec*. Recent study by Madhusoodanan et al. (2011) indicate even lower frequency of enterotoxigenic *S. epidermidis*, since no such isolates were found within more than 200 human bacteraemia isolates.

Current status of knowledge is still not sufficient to describe the genetic processes allowing *S. aureus* and other staphylococci to exchange mobile genetic elements and stably maintain them. The enterotoxin genes detected in some CNS isolates seem to be unstable. According to a phenomenon observed by Park et al. (2011) intensity of PCR amplicons in enterotoxigenic bovine CNS becomes weak or even completely disappear after several passages of the culture. These observations raise a possibility that some genetic elements harboring superantigen-related genes in CNS behave differently than those known from *S. aureus*. We suppose that one of the likely explanations for instability of SE genes in CNS may be inability of the certain mobile genetic elements (MGE) bearing these genes to integrate into host chromosome. That aspect was debated over with regard to *Staphylococcus aureus* Pathogenicity Islands (SaPIs) in *S. aureus* isolates (Novick et al., 2010). It was also experimentally unveiled that MGE instability occurs when problems with segregation to the daughter cells exist (Úbeda et al., 2007).

SEC is among the most common enterotoxins recovered from food poisoning outbreaks worldwide (Kérouanton et al., 2007; Kitamoto et al., 2009). The *sec* gene encoding enterotoxin C was reported as the most frequently occurring within *S. aureus* recovered from milk-related environments (Gutierrez et al., 1982; Scherrer et al., 2004). On the basis on

their antigenic properties SECs fall into several serological variants (SEC_1, SEC_2, SEC_3, SEC_{ovine}, SEC_{bovine}) that display high sequence similarity (Marr et al., 1993). Data on the distribution of SEC subtypes in *S. aureus* related to food-borne outbreaks are scarce; however, in the study performed by Hsiao and colleagues (2003) SEC_3 was shown to dominate within *S. aureus* isolates derived from food-borne outbreaks in Taiwan. Nucleotide sequence of our sec_{epi} gene shares 100% identity with *S. epidermidis* FRI909 *sec*, and it seems to be unique to *S. epidermidis* as the alignment with all available *S. aureus sec* sequences reveals multiple nucleotide substitutions located in sequences encoding both signal peptide and mature protein. Alignment of translated 239-amino acid sequence of mature SEC_{epi} with known *S. aureus* orthologues showed its highest similarity with SEC_3 with 9 amino acids different, and highest disparity with SEC_{ovine}, with 18 amino acid substitutions in mature protein.

Some amino acid substitutions identified in SEC_{epi} are located in regions that are conservative among known *S. aureus* SEC (Table 3). However, based on current knowledge it cannot be definitely resolved whether these mutations can alter biological activity of SEC_{epi} if referred to *S. aureus* SECs. Data on structure/function relations in SE family in many cases are still insufficient to precisely identify residues involved in mitogenic activity of SEs (Zhang and Rogers, 2013). Moreover, site-specific mutagenesis revealed substantial differences within SEs in term of epitopes responsible for mitogenic activity (Briggs et al., 1997).

A disulphide loop comprising from 9 to 19 amino-acids was reckoned to be crucial in SE-mediated emesis (Le Loir et al., 2003). It was identified between 93-110 position in mature SEC_3 (Leder et al., 1998). None of cysteine in that region is substituted in $SEC_{epi.}$ Only one mutation appears in SEC_{epi}, namely G106S, in conserved region in known *S. aureus* SEC.

Staphylococcal pathogenicity islands belong to a group of phage-inducible chromosomal islands (PICIs) occurring in gram-positive bacteria (Novick et al., 2010). Most of them carry genes for one or more superantigenic toxins, besides the subset of genes that allows them to take the advantage of the temperate phages they parasitize (Maiques et al., 2007; Tormo et al., 2008). SaPIs horizontal transfer has already been unequivocally proven (Lindsay et al., 1998). Genome sequencing of *S. epidermidis* 4S isolate revealed that it carries 21,426-bp fragment flanked by DR sequences. This element is almost identical to the composite genomic island designated SePI, previously described by Madhusoodanan et al. (Madhusoodanan et al., 2011) in *S. epidermidis* FRI909 isolate. We have noticed only 6 nucleotide differences in 5 ORF's; however, two of them were located in genes encoding for proteins involved in classical excision-replication-integration pathway, i.e. integrase and transposase (Maiques et al., 2007). Up to date multiple reports on CNS possessing genes homologous to *S. aureus* enterotoxins have appeared (as reviewed by Podkowik et al. (2013)), nonetheless the knowledge about their genetic context is still in its infancy. It may be noteworthy that SePI in both sequenced *S. epidermidis* isolates is larger than most of *S. aureus* SaPIs, that were shown to average 14-17 kb in length (Novick and Subedi, 2007), what may be a reason for the fail in attempts to mobilize SePI, as shown by Madhusoodanan et al. (2011). Dwelling on this issue aforementioned researchers postulate that disruption of ORFs implicated in formation of small SePI phage particles caused by the insertion of

composite SePI elements resulted in inability of SePI-specific transfer (SPST) induction. However, possibility of classical generalized transduction is not excluded, as after mitomycin C treatment the full sized phage particles were observed in culture lysates (in this experiment PCR for toxin genes performed on phage DNA yielded negative results). The primary pending question that should be posed is what factors enabled the stabilization of SePI in *S. epidermidis* chromosome.

The global catalogue of *S. epidermidis* diversity, as sampled by the international MLST database, currently consists of less than 600 sequence types (STs). Our isolate represents a previously unknown ST (allelic profile: 49-3-9-5-NEW-4-4) that is similar to ST561 (allelic profile: 49-3-9-5-8-4-4). By clustering methods, both ST561 and *S. epidermidis* 4S are assigned to the same genetic cluster, GC4. *S. epidermidis* FRI909 belongs to a different, unique ST (allelic profile: 28-16-NEW-5-3-22-56) that has some similarities to ST441 (allelic profile: 28-16-5-5-3-19-49). This ST441 is represented by *S. epidermidis* strain AK18W isolated in 2012 from bovine milk in Bangalore, India (http://sepidermidis.mlst.net/).By clustering methods, both ST441 and *S. epidermidis* FRI909 are also assigned to GC4. Thus, despite the different MLST allelic profiles, the analysis of nucleotides places both SEC-producing strains into the same genetic cluster, GC4. The question of the normal habitat of GC4 isolates requires a close investigation, considering that some of its isolates produce high levels of SEC under certain conditions.

qRT-PCR, western blotting and ELISA that we have performed unambiguously confirmed that SePI-located *sec* gene is expressed in *S. epidermidis* 4S isolate. Based on qRT-PCR data for *S. epidermidis* 4S, with high post-exponential *sec* transcript increase, we reckon that SEC_{epi} production remains under control of the same global regulator (*agr*) as the *S. aureus* SEC.

SEC production was assayed in culture supernatants of studied strains after 24 and 48h cultivation in BHI broth, milk and meat juices. The choice of milk as a food model was due to the fact that *S. aureus* is a common pathogen contaminating milk-related environments, and that milk was already used in studies examining SE production (Valihrach et al., 2013, 2014). Thus far, the production of SEC in milk was reported by Valihrach and colleagues (2014), indicating a low SEC production in milk by 14 *S. aureus* isolates studied if compared with SEC production in laboratory media. This remains consistent with our data obtained for *S. epidermidis* 4S and food-derived *S. aureus* 175 isolate. On the contrary, our reference *S. aureus* strains, i.e., FRI137 and FRI913, elaborated several dozen-fold higher amounts of SEC in milk compared to *S. epidermidis* 4S isolate. However, our results on production of various enterotoxins in milk and microbial broths by a number of food-derived *S. aureus* strains allowed us to classify *S. aureus* FRI137 and FRI913 as enterotoxin hyper-producers (authors' unpublished data). It can be thus possible that some *S. aureus* strains can secrete less enterotoxin to milk than to microbial media, and this is also the case of our *S. epidermidis* isolate. On the contrary, examination of microbial broth culture supernatants revealed that *S. epidermidis* 4S is able to elaborate several fold higher amounts of SEC than isolates studied by Valihrach et al. (2014), and even more than *S. aureus* FRI137, FRI913, and 175. Since enterotoxigenic *S. epidermidis* was isolated from meat product SEC production was also assessed in meat juices. We revealed that *S. epidermidis* 4S secreted less

SEC to meat juices than to BHI, but much more than to milk. It produced highest amounts of SEC in beef juice as compared to tested *S. aureus* strains. In pork meat juice *S. epidermidis* 4S was able to produce almost 2 μg/mL of SEC after 48-hour culture.

In most cases SEs produced by *S. aureus* in food involved in SFP outbreaks were detected between 5 and 100 ng/mL (Bergdoll, 1989; Evenson et al., 1988). Data presented by Jay (1992) indicate that even 1 ng/g of SE in food is enough to cause food poisoning symptoms.

This implies that amounts of SEC secreted by *S. epidermidis* 4S to milk (running up to 9 ng/mL after 48 hour) and especially to meat juices (almost 3 μg/mL after 48 hours) are sufficient to act as food safety hazard.

We demonstrated that *S. epidermidis* occurring in food bears a genetic element encoding an orthologue to *S. aureus* SEC. SEC_{epi} can be produced both in microbial broth, meat juices and in milk. Regarding that only enterotoxins produced by *S. aureus* are officially tracked in food in EU the ability of enterotoxin production by *S. epidermidis* pose real risk for food safety.

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Highlights

- *S. epidermidis* 4S isolate from food stably harbor *sec* and *sel* genes
- enterotoxin genes are on staphylococcal pathogenicity island in *S. epidermidis* 4S
- genomic location of *S. epidermidis* 4S SePI is the same as in *S. epidermidis* FRI909
- *S. epidermidis* 4S is genetically different from *S. epidermidis* FRI909
- SEC_{epi} is secreted by *S. epidermidis* 4S to milk, meat juice and to microbial broth

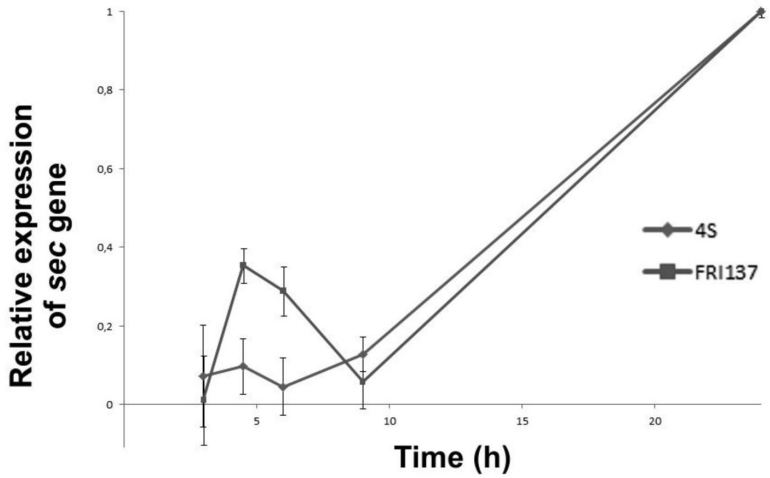

Figure 1.
RNA expression of *S. epidermidis* 4S sec_{epi} and *S. aureus* FRI137 *sec* genes.

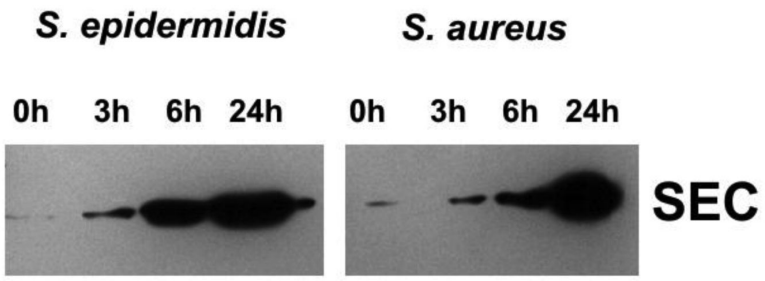

Figure 2.

Western blotting of culture supernatants collected from *S. epidermidis* 4S and *S. aureus* FRI137 grown for 0, 3, 6 and 24 hours in BHI broth. Five-μg portions of protein were loaded per well in SDS-PAGE. Western immunoblots were probed with rabbit anti-SEC antibodies (Acris, Herford, Germany).

Table 1

Reference *S. aureus* strains used for enterotoxin genes detection.

Strain	Enterotoxin gene content
FRI913	*sea, sec, see, sek, sel, tst*
FRI137	*sec, seh, sel, sem, sen, seo, seg, sei, selu*
CCM5757	*seb, sek*
A900322	*sep, sem, sen ,seo, seg, sei,*
FRI1151m	*sed, selj, ser*

Table 2

Nucleotide mismatches and corresponding amino acid alternations in *S. epidermidis* 4S vs. *S. epidermidis* FRI909.

ORF[a]	Predicted function	Nucleotide		Mismatch position[b]	Amino acid		Strand
		4S	FRI909		4S	FRI909	
2	Integrase	A	G	21,012	I	V	+
11	Transposase	T	G	13,206	D	A	−
15	Hypothetical *S. aureus* protein	C	T	9,125	D	N	−
20	Hypothetical *S. aureus* protein	T	C	6,202	P	P	−
20	Hypothetical *S. aureus* protein	T	C	6,099	T	A	−
22	Hypothetical protein	G	T	4,439	W	L	+

[a] ORFs numeration positions are based on their order in *S. epidermidis* 4S SePI, GenBank Accession KT845956

[b] ORFs mismatches positions are based on their order in *S. epidermidis* 4S SePI, GenBank Accession KT845956

Table 3

Comparison of predicted 266 amino acid sequences of SEC_1, SEC_2, SEC_3,SEC_{ovine}, SEC_{bovine}, and SEC_{epi}

Enterotoxin	Strain	Variable positions in predicted amino acid SEC sequences																															aa difference		
		2	4	5	7	9	15	20	21	22	34	37	41	43	47	49	53	58	66	81	86	99	102	121	133	154	160	192	218	243	245	249	total	sp	mp
SEC_3	*S. aureus* Mu3	-	-	-	I	-	-	-	S	T	-	-	-	-	-	-	-	-	-	S	K	-	K	-	G	-	V	-	N	M	N	T	12	3	9
SEC_2	*S. aureus* Tokyo12381	N	S	R	I	C	-	L	-	T	T	E	-	-	-	-	-	-	M	S	K	-	K	-	G	-	-	-	N	M	N	T	18	7	11
SEC_1	*S. aureus*	N	S	R	I	C	-	L	-	T	T	-	A	K	-	E	V	-	-	S	K	G	K	-	G	-	-	-	N	M	N	T	21	7	14
SEC_{ovine}	*S. aureus* ED133	N	S	R	I	C	S	L	-	T	T	E	A	K	T	E	V	R	-	S	K	-	K	C	G	-	-	S	N	M	N	T	26	8	18
SEC_{bovine}	*S. aureus* RF122	N	S	R	I	C	-	L	-	T	T	E	A	K	T	E	V	R	-	S	K	-	K	-	G	K	-	-	N	M	N	T	24	7	17
SEC_{epi}	*S. epidermidis* 4S/FRI909	Y	R	L	V	R	A	I	F	I	M	D	S	E	L	G	Y	H	K	N	N	D	N	Y	S	N	I	N	S	I	K	M	-	-	-
		Signal peptide																																	

sp - signal peptide, mp - mature protein

Accession numbers (GenBank): SEC_3 - *S. aureus* Mu3: AP009324.1 (complete genome), SEC_2 - *S. aureus* Tokyo12381: AB860418.1 (SaPITokyo12381, complete sequence), SEC_1: KF386012.1 (enterotoxin type C1 precursor gene), SEC_{ovine}- *S. aureus* ED133: NC_017337.1 (complete genome), SEC_{bovine}- *S. aureus* RF122: NC_007622.1 (complete genome)

Table 4

SEC production and bacterial growth in BHI broth, milk, beef and pork meat juices assessed with ELISA.

	BHI broth				Milk				Beef meat juice				Pork meat juice			
	24 h		48 h		24 h		48 h		24 h		48 h		24 h		48 h	
Isolate	**SEC [ng/mL]**	**Log CFU/mL**	**SEC [ng/mL]**	**Log CFU/mL**	**SEC [ng/mL]**	**Log CFU/mL**	**SEC [ng/mL]**	**Log CFU/mL**	**SEC [ng/mL]**	**Log CFU/mL**	**SEC [ng/mL]**	**Log CFU/mL**	**SEC [ng/mL]**	**Log CFU/mL**	**SEC [ng/mL]**	**Log CFU/mL**
4S	14,001 ± 2,651	9.0 ± 0.2	35,626 ± 7,892	9.4 ± 0.1	6 ± 1	8.9 ± 0.7	9 ± 2	7.8 ± 0.1	2,283 ± 192	6.7 ± 0.3	2,979 ± 211	7.7 ± 0.2	1,256 ± 44	8.4 ± 0.2	1,877 ± 121	7.3 ± 0.2
175 *	236 ± 47	9.0 ± 0.2	371 ± 36	9.5 ± 0.4	2 ± 1	8.5 ± 0.3	8 ± 1	7.8 ± 0.3	112 ± 18	6.0 ± 0.1	601 ± 2	7.9 ± 0.2	133 ± 8	7.5 ± 0.3	263 ± 18	8.4 ± 0.1
FRI137	296 ± 41	8.4 ± 0.5	30,087 ± 621	9.3 ± 0.4	131 ± 15	8.6 ± 0.4	2,319 ± 76	9.3 ± 0.3	9 ± 1	9.1 ± 0.3	15 ± 1	9.1 ± 0.4	27 ± 2	8.4 ± 0.2	43 ± 3	7.3 ± 0.3
FRI913	4,134 ± 315	9.7 ± 0.2	11,969 ± 3,793	9.8 ± 0.1	85 ± 10	9.0 ± 0.1	332 ± 62	8.7 ± 0.1	471 ± 25	9.5 ± 0.1	2,428 ± 237	9.1 ± 0.1	3,210 ± 243	8.7 ± 0.1	5,736 ± 232	9.7 ± 0.1

* wild-type *S. aureus* isolate derived from food of animal origin. Positive for *sec, sel, sep,* enterotoxin genes.