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Impact of co-cultivation with *Enterococcus faecalis* over growth, enterotoxin production and gene expression of *Staphylococcus aureus* in broth and fresh cheeses

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ABSTRACT

Biocontrol of *Staphylococcus aureus* by lactic acid bacteria can be considered as a feasible alternative to the use of chemicals in foods, but the mechanisms underlying this antagonistic interaction remains poorly understood. This study aimed to evaluate the impact of a LAB species (*Enterococcus faecalis*) over the growth, enterotoxin production and gene expression of *S. aureus* under experimental conditions. *E. faecalis* 41FL1 and *S. aureus* ATCC 29213 were inoculated isolated and together in brain heart infusion (BHI) at 30 °C and in a fresh cheese model at 15 °C: microbial populations were monitored by culture plating, production of classical staphylococcal enterotoxins (SEs) was verified by an ELISA assay, expression of *S. aureus* genes (virulence, transcriptional regulation and central carbon metabolism) was investigated by quantitative real-time PCR, and pH and contents of water-soluble metabolites in both matrices were measured. *S. aureus* growth was inhibited in co-cultures assays, with a 2.02-log reduction in BHI and a 3.39-log reduction in cheese model compared to respective single cultures. Classical SEs were detected in *S. aureus* single culture assays (BHI and cheese), in BHI inoculated with both strains after 48 h of incubation, but not detected in co-inoculated cheeses. pH in all matrices containing *E. faecalis* reached lower values than in matrices containing *S. aureus* alone, due to lactate production by *E. faecalis*. Expression of genes coding for transcription regulators (*ccpA* and *rex*) and enzymes involved in central carbon metabolism (*alsD* and *citZ*) was mostly upregulated in co-inoculated cheeses, whereas expression of several virulence determinants (*agrC, hld, hla, entA* and *spa*) was strongly downregulated. This study provides relevant data on the behaviour of *S. aureus* in the presence of competing microbiota and support the use of controlled population dominance by LAB as an effective biopreservation strategy to ensuring food safety.

1. Introduction

Staphylococcal food poisoning (SFP) results from the consumption of foods containing preformed thermostable staphylococcal enterotoxins (SEs) secreted by *Staphylococcus aureus* enterotoxigenic strains in sufficient amounts to induce symptoms such as nausea, abdominal cramps, and emesis (Fisher et al., 2018). Foods implicated in SFP vary among countries due to differences in dietary habits (Hennekinne et al., 2012; Scallan et al., 2011), but milk and cheeses are among the most frequent sources of SFP outbreaks worldwide (Asao et al., 2003; do Carmo et al., 2002; Johler et al., 2015). *S. aureus* strains isolated from dairy products often harbour multiple SEs genes (Arcuri et al., 2010; Viçosa et al., 2013), which may constitute a threat to human health if strains encounter conditions enabling growth and SE production (Argudín et al., 2010).

A great fraction of the naturally-occurring microbiota of raw milk and dairy products is constituted by lactic acid bacteria (LAB), a heterogeneous group of bacteria whose activity has been demonstrated to inhibit a broad range of food-spoilage and pathogenic bacteria (Charlier et al., 2009). LAB antagonistic effect over *S. aureus* in dairy-mimicking models has been linked to several aspects: bacteriocin- (Hamama et al., 2002) and hydrogen peroxide-production (Delbes-Paus et al., 2010), competition for nutrients (Alomar et al., 2008; Charlier et al., 2008; Iandolo et al., 1965), or a combined effect of environmental stressors (Delbes et al., 2006; Viçosa et al., 2018). Such inhibitory effect is influenced by the composition of the surrounding medium or food matrix

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(Alomar et al., 2008; Cretenet et al., 2011; Delbes et al., 2006), the initial inoculum size of LAB and of *S. aureus* (Gómez-Lucía et al., 1992; Iandolo et al., 1965; Meyrand et al., 1998), and features inherent to certain LAB species or strains (Kao and Frazier, 1966; Perin and Nero, 2014).

In recent years, particular attention has been given to biopreservation, i.e. the use of indigenous or added biological systems with antagonistic activity over undesired microorganisms as a tool to extend food shelf-life and control pathogenic bacteria (Stiles, 1996). Biopreservation using LAB has been a common practice since ancient times, being widely employed in food fermentations (Caplice and Fitzgerald, 1999). The predominance of LAB in the microbiota of certain foods, in particular cheeses, offers a barrier against spoilage and pathogen multiplication, and confers unique sensory characteristics (Jordan et al., 2014; Stiles, 1996). However, for the biopreservation strategy to be successful in foods, it is crucial to understand how target microorganisms, especially pathogens, are affected by the activity of antagonistic microbiota at phenotypical and molecular levels.

In a previous study, we investigated the behaviour of *S. aureus* ATCC 29213 in skimmed milk in co-culture with *Enterococcus faecalis* 41FL1, a LAB strain isolated from an artisanal Italian cheese (Dal Bello et al., 2010; Viçosa et al., 2018). To gain a deeper understanding of the interactions, we evaluated the impact of *E. faecalis* 41FL1 over the production of SEs, growth, and gene expression of *S. aureus* ATCC 29213 in laboratory rich medium and miniaturised soft fresh cheeses. The results provided herein add up relevant evidences to the current body of knowledge focusing on the LAB-foodborne pathogen interaction and support the use of LAB for controlling *S. aureus* growth and virulence in foods, especially cheeses.

2. Material & methods

2.1. Bacterial strains

S. aureus ATCC 29213 and *E. faecalis* 41FL1 were used in this study. *E. faecalis* 41FL1 was identified by 16S rRNA gene sequencing and *S. aureus* ATCC 29213 was characterized by whole genome sequencing (GenBank accession number: PRJNA344949). Both strains were kept in brain heart infusion (BHI, Sigma-Aldrich, St. Louis, USA) with 20% glycerol at −80 °C and activated in BHI or sterile skimmed milk (Sigma-Aldrich) at 30 °C for 18 h prior to each experiment.

2.2. Interaction assays

BHI broth (Sigma-Aldrich) was inoculated with: 1) *S. aureus* ATCC 29213 alone (10^3 colony forming units [CFU/mL]), 2) *E. faecalis* 41FL1 alone (10^6 CFU/mL), and 3) *S. aureus* ATCC 29213 (10^3 CFU/mL) and *E. faecalis* 41FL1 (10^6 CFU/mL). Inoculum size of *E. faecalis* was chosen considering LAB initial load in most cheeses (10^6–10^7 CFU/mL). Level of *S. aureus* used in our experiments was based on maximum contamination level in cheeses established by the European Union for coagulase-positive staphylococci (EC n. 2073/2005). BHI was chosen for being suitable to the growth of both strains. BHI assays were incubated under static conditions at 30 °C for 48 h. Experiments were reproduced independently at least three times.

A fresh cheese model was produced based on Minas Frescal cheese production (Carvalho et al., 2007). All steps for cheese production were conducted in laboratory environment to avoid external contamination. Raw milk was subjected to tangential microfiltration with a 1.4-μM pore size membrane (Bactocatch®, Tetra Laval Co., Pully, Switzerland) and divided in 3 sets of 3 L, being inoculated with: 1) *S. aureus* ATCC 29213 alone (10^3 CFU/mL), 2) *E. faecalis* 41FL1 alone (10^6 CFU/mL), and 3) *S. aureus* ATCC 29213 (10^3 CFU/mL) and *E. faecalis* 41FL1 (10^6 CFU/mL). Then, 300 μL of 0.22 μm-filtered concentrated recombinant chymosin (Lacto-Lab, Itanhaém, BR) diluted in 5 mL of sterile distilled water were incorporated into each vat. Coagulation occurred within 30–40 min at 34 °C. The curd was cut using a sterile knife and transferred into cheese moulds using a sterile ladle. Cheeses were kept closed in sterile storage trays at 30 °C for 6 h to enable whey separation and gravitational pressing. Cheeses were salted using sterile powdered NaCl, unmoulded and placed at 15 °C for 21 days in a clean storage tray. Each cheese set yielded five analytical units of approximately 60 g each. The experiment was repeated three times independently.

2.3. Microbiological analysis

Aliquots of BHI cultures of *S. aureus* and *E. faecalis* were obtained after 4, 7, 12, 24 and 48 h of incubation. Cheese units were obtained after 1, 3, 7, 15 and 21 days of storage. Samples were ten-fold diluted in NaCl 0.85%, and selected dilutions were plated in duplicate and spread plated in BHI (Sigma-Aldrich) for *E. faecalis* 41FL1 enumeration, and Baird-Parker Rabbit Plasma Fibrinogen agar (BP-RPF) (bioMérieux, Marcy-l'Étoile, France) for *S. aureus* ATCC 29213 enumeration. Plates were incubated at 37 °C for 48 h. Colonies from BHI were selected and subjected to catalase test to allow presumptive enumeration of *E. faecalis* 41FL1. The obtained counts were presented in CFU per mL or g and converted to $\log_{10}$ for statistical analysis.

2.4. Enterotoxin detection

At the same intervals set for bacterial enumeration, samples of BHI and cheese were collected and qualitatively evaluated for the presence of SE using Ridascreen SET total (R-Biopharm, Darmstadt, Germany) following manufacturer's instructions.

2.5. Determination of soluble metabolites and pH measurements

Water-soluble metabolites present in samples were determined by high performance liquid chromatography (HPLC) using the protocol described by Bertolino et al. (2011). Firstly, 5 mL of BHI or 5 g of cheeses samples were added to 20 mL of 0.013 N H_2SO_4 (mobile phase) and homogenized for 30 min in a horizontal shaker (Asal, Milan, Italy) at 100 oscillation/min. After centrifugation (5 min, 10,000 × *g*, 10 °C), the supernatant was filtered through a 0.2-μm polypropylene membrane filter (VWR, Milan, Italy). The HPLC system (Finnigan™ SpectraSYSTEM™; Thermo Fisher Scientific, Waltham, USA) consisted of an isocratic pump (P4000), a multiple autosampler (AS3000) fitted with a 20 μL loop, an UV detector (UV100) set at 210 nm and a refractive index detector RI-150. Analysis were performed isocratically, at 0.8 mL min^{-1} and 65 °C, with a 300 × 7.8 mm i.d. cation exchange column (Aminex HPX-87H) equipped with a Cation H^+ Microguard cartridge (Bio-Rad Laboratories, Hercules, USA). Two replicates were examined for each biological replicate. Data treatments were carried out using ChromQuest™ 5.0 chromatography data system software (Thermo Fisher Scientific). Analytical grade reagents (Sigma-Aldrich) were used as standards.

pH measurements were collected with a pHmeter (Crison, Modena, Italy) at the same intervals described above.

2.6. Expression of S. aureus genes involved in virulence and carbon metabolism

For RNA extraction, cells from 1 mL of BHI samples were harvested in triplicates after 7, 24 and 48 h of incubation at 30 °C; RNA was extracted using the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre, Madison, USA) following the manufacturer's instructions. From cheeses, samples of 2 g were homogenized with 8 mL of 2% (w/v) trisodium citrate solution, and the obtained cells were harvested by centrifugation (6000 × *g*, 5 min, 4 °C); cell pellets were resuspended in 2 mL of TE buffer and enzymatic lysis was performed using 250 μL of lysozyme (20 mg/mL) (Sigma-Aldrich) and 250 μL of lysostaphin

(1 mg/mL) (Sigma-Aldrich), followed by incubation at 37 °C for 30 min. Lysed cells were centrifuged (6000 ×*g*, 5 min, 4 °C) and 2 mL of phenol:chloroform solution (5:1) (Sigma-Aldrich) were added to the samples. After centrifugation (12,000 ×*g*, 20 min, 4 °C), the aqueous phase was recovered and transferred to a clean tube with 1:1 (v/v) of isopropanol (Sigma-Aldrich). Tubes were gently mixed, centrifuged (12,000 ×*g*, 5 min, 4 °C) and supernatants were discarded. An equal volume of 70% ethanol (Sigma-Aldrich) was added to the tubes, which were inverted multiple times and centrifuged (12,000 ×*g*, 5 min, 4 °C). The supernatants were discarded and the resultant pellets were allowed to air-dry for 30 min. Pellets were resuspended in 30 µL of DEPC-treated water (Sigma-Aldrich) and incubated at 60 °C for 10 min.

RNA was treated with Ambion® TURBO DNA-free™ kit (Thermo Fisher Scientific) to remove genomic DNA (gDNA). RNA integrity was evaluated by agarose gel electrophoresis and quantification was determined in Agilent 2200 Tape Station Nucleic Acid System (Agilent Technologies, Santa Clara, USA).

Eleven *S. aureus* genes (*codY*, *ccpA, alsD*, *citZ, rex, sigB, agrC*, *hld*, *hla, spa, sea*) were selected for real-time quantitative reverse transcription PCR (RT-qPCR). After a preliminary evaluation of expression stability of several candidates, *mqo2* was selected as reference gene. Target genes were selected considering their role in virulence and central carbon metabolism (Table 1). Oligonucleotides used in reverse transcription (RT) and RT-qPCR reactions were designed using Primer-BLAST (Ye et al., 2012) based on the genome sequence of *S. aureus* ATCC 29213 (Table 1). Confirmation of in silico specificity of oligonucleotides sequences was performed with UGENE software version 1.26.1 (Okonechnikov et al., 2012) and BLAST (Altschul et al., 1990) against NCBI database. Absence of gDNA in RNA samples was verified by RT-PCR prior to cDNA synthesis. Gene-specific RT was performed on 100 ng of RNA with 1 µL of reverse primer (100 µM) and ultrapure water in a 10 µL final volume reaction. The mix was treated at 75 °C for 5 min to enable RNA denaturation and placed on ice for 10 min. Five microliters of M-MLV RT Buffer (1 ×), 5 µL of dNTPs (10 µM each), 1 µL of M-MLV Reverse Transcriptase (8 U/µL) and 0.6 µL of RNasin ribonuclease inhibitor (20 U/µL) were added to the mix for a final volume of 25 µL by addition of ultrapure water. RT reaction was carried out at 42 °C for 1 h in a Biorad DNA Engine thermal cycler (Bio-Rad) and cDNA was stored at −20 °C. RT-qPCR reactions were performed on respective cDNAs in a final volume of 20 µL using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) in a MJ Research PTC-200 DNA Engine® Peltier Thermal Cycler (Bio-Rad). Thermal cycling parameters included an initial denaturation at 98 °C for 30 s, followed by 40 cycles at 95 °C for 15 s and 30 s at the annealing temperature optimized for each primer pair (Table 1). No-template and no-RT served as negative controls on each run. Melting curve analysis confirmed the specificity of amplification (50.0 °C to 95.0 °C, reading every 1.0 °C and holding for 5 s). Efficiency of amplifications was calculated from 10-fold dilutions of template DNA. All reactions were performed in triplicates for each sample. Mean values were calculated from obtained results and used to determine the threshold cycle (Ct). Relative changes in gene expression were calculated using the $2_T^{-\Delta\Delta C}$ method (Livak and Schmittgen, 2001). R^2 calibration curve slopes were ≥0.99. Fold change for each gene was calculated considering the single culture as reference. Genes showing a $\log_2$ fold change (LFC) ≥ +1 or ≤ −1 were considered differentially expressed.

2.7. Statistical analysis

The obtained data (microbiological counts, water soluble metabolites, pH and gene expression) were subjected to one-way analysis of variance (ANOVA) with Tukey's honest significant difference post-hoc test. Differences in mean counts of microbial populations in single and co-culture experiments were assessed by a Student's *t*-test. Statistical analyses were performed using Statistica software version 6 (Statsoft, Tulsa, OK, USA) with significance set at $p < 0.05$.

3. Results

Microbiological counts obtained in BHI and cheese samples are detailed in Tables 2 and 3. Kinetics of *E. faecalis* population showed no significant differences between single and co-culture in both tested matrices (Tables 2 and 3). In BHI, counts of *S. aureus* in co-culture increased during the initial 24 h, reaching a final population 2.02-log

Table 1
Sequences of primers for real-time RT-qPCR analysis of selected *S. aureus* genes.

Primer	Gene	Gene product	Biological process	Main role	Sequence (5′–3′)[a]	Product length (bp)	Annealing temperature (°C)
mqo2_f	*mqo2*	Malate:quinone oxidoreductase	Carbohydrate metabolism	Oxaloacetate production from malate	GAAGTTGAAGCCCCTGGTGA	107	53.7
mqo2_r					ACACCTGAACACGGTAAAGGA		
alsD_f	*alsD*	Alpha-acetolactate decarboxylase	Carbohydrate metabolism	Acetoin production from acetolactate	ACCAACGACTGAACCTTTGACA	93	59.4
alsD_r					CCGGCTCAAGAACCACCTTAT		
citZ_f	*citZ*	Citrate synthase	Carbohydrate metabolism	Citrate production from acetyl-CoA and oxaloacetate	ACACGATGACCAAAGCCCAT	135	65.0
citZ_r					AGGGCCATTACATGGTGGTG		
codY_f	*codY*	GTP-sensing transcriptional regulator CodY	Transcriptional regulator	Repression of branched-chain amino acids synthesis	GAAGAACTTGGCGGTACGGA	125	59.4
codY_r					CGTGATTCAATTACACCAGCACT		
ccpA_f	*ccpA*	Catabolite control protein A	Transcriptional regulator	Catabolite repressor or activator, depending on carbon or amino acid source	TCAAGTCCACGAGCAAGTTGT	190	62.0
ccpA_r					CGCGTGTTGTTAATGGGAACC		
rex_f	*rex*	Redox-sensing transcriptional repressor Rex	Transcriptional regulator	Activation of fermentative pathways	GTACTTGCACATCTGAAGGCG	79	64.1
rex_r					GAACTCGTCCAAGCTGGTGT		
sigB_f	*sigB*	RNA polymerase sigma factor B	Transcriptional regulator	Involved in environmental stress response	CCGATACGCTCACCTGTCTC	149	65.0
sigB_r					ATGGGGCAACAAGATGACCA		
agrC_f	*agrC*	Accessory gene regulator sensor histidine kinase AgrC	Virulence factor	Involved in quorum sensing and toxin production	TGATGACCCTATCATTCGCGT	142	54.8
agrC_r					ACCACGACCTTCACCTTTAGT		
hld_f	*hld*	Delta-hemolysin	Virulence factor	Toxin production	AATTTGTTCACTGTGTCGATAATCC	80	51.4
hld_r					GGAGTGATTTCAATGGCACAAG		
hla_f	*hla*	Alpha-hemolysin	Virulence factor	Toxin production	GCGAAGTCTGGTGAAAACCC	130	61.8
hla_r					CTTGGAACCCGGTATATGGCA		
spa_f	*spa*	Immunoglobulin G binding protein A precursor	Virulence factor	Immune system evasion	GCATGGTTTGCTGGTTGCTT	173	54.8
spa_r					GAAGACGGCAACGGAGTACA		
sea_f	*sea*	Staphylococcal enterotoxin type A	Virulence factor	Toxin production	TCCCCTCTGAACCTTCCCAT	91	51.4
sea_r					TCAGGAGTTGGATCTTCAAGCA		

[a] All primers sequences and qRT-PCR protocol reactions were designed in the present study.

Table 2
Mean counts with standard deviations (MC ± SD) of *S. aureus* ATCC 29213 and *E. faecalis* 41FL1 populations (log_{10} CFU/mL) and production of staphylococcal enterotoxins in single and co-culture experiments in BHI broth.

Incubation time (hours)	*S. aureus*				*E. faecalis*	
	Single culture		Co-culture		Single culture	Co-culture
	MC ± SD	SE production	MC ± SD	SE production		
0	2.93 ± 0.16[a]	nd	2.95 ± 0.35[a]	nd	6.31 ± 0.02[a]	6.38 ± 0.04[a]
4	3.66 ± 0.89[a]	+	4.48 ± 0.21[a]	nd	7.07 ± 0.12[a]	7.05 ± 0.24[a]
7	5.18 ± 1.58[a]	+	5.22 ± 0.10[a]	nd	9.02 ± 0.07[a]	8.87 ± 0.29[a]
12	7.05 ± 1.36[a]	+	5.68 ± 0.06[a]	nd	9.31 ± 0.02[a]	9.26 ± 0.11[a]
24	8.40 ± 0.96[a]	+	6.32 ± 0.01[b]	nd	9.31 ± 0.02[a]	9.29 ± 0.03[a]
48	8.26 ± 0.47[a]	+	6.24 ± 0.02[b]	+	9.23 ± 0.04[a]	9.24 ± 0.03[a]

[a,b]Different letters in the same row indicate difference at 95% level of significance; nd: not detected.

lower compared to single culture (Table 2). In cheese, the dynamic of *S. aureus* growth in the presence of *E. faecalis* was significantly different ($p < 0.05$) from single culture since day 1, resulting in a 3.39-log decrease in final population (Table 3). Considering *S. aureus* enterotoxigenic ability (Tables 2 and 3), SEs were detected in *S. aureus* single culture in BHI and cheese, as well as in co-inoculated BHI after 48 h of incubation, but no SE were produced at detectable levels in co-inoculated cheeses throughout the storage period.

Data concerning pH and HPLC analysis of BHI and cheeses are presented, respectively, in Tables 4 and 5. pH values of *E. faecalis* in BHI as single and co-culture were significantly lower ($p < 0.05$) than those with *S. aureus* alone, which can be linked to the increasing concentrations of lactic acid in the corresponding samples (Table 4). Similarly, pH in cheeses where *E. faecalis* was inoculated alone or in combination with *S. aureus* differed remarkably from pH values of cheeses containing only *S. aureus* (Table 5). HPLC analysis of BHI samples showed a progressive accumulation of lactate and acetate in co- and single cultures throughout the incubation period, whereas only traces of citrate and pyruvate were present (Table 4). Conversely, HPLC analysis of cheese samples was more complex, reporting the presence of several metabolites (Table 5). Lactose concentration rapidly decreased in cheeses containing *E. faecalis* compared to cheeses with *S. aureus* alone, which was followed by higher concentrations of galactose, a by-product of lactose degradation. Lactate was significantly ($p < 0.05$) more abundant in cheeses where *E. faecalis* was added compared to cheese with *S. aureus* alone. Citrate and acetate concentrations in cheeses where *S. aureus* was inoculated alone were significantly higher ($p < 0.05$) in comparison to the co-cultivation essay, whereas acetoin was present in higher amounts in co-inoculated cheeses.

The expression of *S. aureus* selected genes in co-culture compared to single culture in BHI and cheese are reported, respectively, in Figs. 1 and 2. In BHI, the expression of *codY* and *ccpA*, coding for the metabolite responsive regulators CodY and CcpA, along with *rex* and *citZ*, encoding respectively the redox responsive regulator Rex and the TCA cycle enzyme citrate synthase, was constantly upregulated in *S. aureus* in co-culture (Fig. 1). The expression of *agrC* and *hld*, found within the *agr* (*a*ccessory *g*ene *r*egulator) locus, a global regulator of virulence genes in *S. aureus*, was decreased in co-cultured BHI, as well as for *sigB* and *sea*, encoding respectively the RNA-polymerase sigma factor B and the staphylococcal enterotoxin A (SEA) (Fig. 1). *S. aureus* gene expression in cheese shared similarities with the gene expression pattern depicted in BHI, showing a downregulation of virulence genes and upregulation of transcriptional regulators (Fig. 2). However, remarkable differences were found concerning the expression of *alsD* and *citZ*. Interestingly, *alsD*, which codes for the alpha-acetolactate decarboxylase involved in the biosynthesis of acetoin, along with *sigB*, were found to be upregulated in co-cultured cheeses, whereas expression of *citZ* was reduced compared to BHI (Fig. 2).

4. Discussion

In the present study, a strong inhibitory effect of *E. faecalis* 41FL1 over *S. aureus* ATCC 29213 was observed in BHI and soft fresh cheese, affecting primarily *S. aureus* growth, but also altering its enterotoxigenic ability and the expression of certain genes involved in central metabolism, transcriptional regulation and virulence.

Previous studies have assessed the inhibitory potential of certain LAB species over the growth and enterotoxin production of *S. aureus* in different media (Iandolo et al., 1965; Noleto et al., 1987; Peterson et al., 1962). These early reports demonstrate that the degree of antagonistic effect over *S. aureus* growth in mixed culture with LAB is influenced by the relative proportion of *S. aureus* in the initial bacterial population. In our study, using a 3-log difference in initial inoculum sizes between the chosen strains, *S. aureus* growth was repressed in both matrices: increase in numbers of both bacterial populations was verified in BHI, with an early arrest of *S. aureus* growth in co-culture (Table 2), whereas counts of *S. aureus* in co-inoculated cheese did not increase over 5.8 log, probably due to repression during cheesemaking (Table 3). Thus, poor competitiveness of *S. aureus* in conditions where large numbers of accompanying microbiota are usually found, such as in raw and

Table 3
Mean counts with standard deviations (MC ± SD) of *S. aureus* ATCC 29213 and *E. faecalis* 41FL1 populations (log_{10} CFU/mL) and production of staphylococcal enterotoxins in single and co-culture experiments in miniaturised soft fresh cheeses.

Incubation time (days)	*S. aureus*				*E. faecalis*	
	Single culture		Co-culture		Single culture	Co-culture
	MC ± SD	SE production	MC ± SD	SE production		
1	7.83 ± 0.53[a]	+	5.48 ± 0.36[b]	nd	9.99 ± 0.18[a]	9.80 ± 0.38[a]
3	8.02 ± 0.74[a]	+	5.71 ± 0.20[b]	nd	10.14 ± 0.03[a]	10.06 ± 0.06[a]
7	8.47 ± 0.54[a]	+	5.88 ± 0.27[b]	nd	10.11 ± 0.42[a]	9.08 ± 0.52[a]
15	8.42 ± 0.56[a]	+	5.70 ± 0.25[b]	nd	9.98 ± 0.40[a]	10.07 ± 0.58[a]
21	8.47 ± 0.33[a]	+	5.08 ± 0.62[b]	nd	10.03 ± 0.78[a]	10.05 ± 0.76[a]

[a,b]Different letters in the same row indicate difference at 95% level of significance; nd: not detected.

Table 4
pH and metabolite concentration (g/L) in BHI broth inoculated with *E. faecalis* 41FL1 and *S. aureus* ATCC 29213 alone and in combination during 48 h at 30 °C. (pH of uninoculated BHI: 7.5).

Chemical parameter	Assay	Hours				
		4	7	12	24	48
pH	*E. faecalis*	6.83 ± 0.02[a]	6.24 ± 0.01[a]	5.40 ± 0.01[a]	5.25 ± 0.01[a]	5.26 ± 0.01[a]
	Co-culture	6.83 ± 0.08[a]	6.20 ± 0.05[a]	5.41 ± 0.01[a]	5.28 ± 0.02[a]	5.32 ± 0.02[a]
	S. aureus	7.30 ± 0.00[b]	7.27 ± 0.02[b]	6.91 ± 0.02[b]	6.10 ± 0.02[b]	5.92 ± 0.01[b]
	Significance	*	*	*	*	*
Citric acid	*E. faecalis*	0.06 ± 0.00[a]	0.07 ± 0.01[ab]	0.05 ± 0.00[a]	0.05 ± 0.00[c]	0.04 ± 0.00
	Co-culture	0.09 ± 0.00[c]	0.08 ± 0.00[b]	0.08 ± 0.00[b]	0.04 ± 0.00[b]	0.04 ± 0.01
	S. aureus	0.08 ± 0.00[b]	0.07 ± 0.00[a]	0.06 ± 0.01[a]	0.03 ± 0.00[a]	0.03 ± 0.01
	Significance	*	*	*	*	ns
Pyruvic acid	*E. faecalis*	0.01 ± 0.00	0.02 ± 0.00[b]	0.01 ± 0.00	nd	nd
	Co-culture	0.01 ± 0.00	0.01 ± 0.00[a]	0.01 ± 0.00	nd	nd
	S. aureus	nd	nd	0.01 ± 0.00	nd	nd
	Significance	ns	*	ns	ns	ns
Lactic acid	*E. faecalis*	0.12 ± 0.01[b]	0.41 ± 0.02[b]	1.90 ± 0.02[b]	2.89 ± 0.03[b]	3.44 ± 0.10[b]
	Co-culture	0.16 ± 0.02[c]	0.36 ± 0.05[b]	1.87 ± 0.09[b]	2.93 ± 0.05[b]	3.38 ± 0.08[b]
	S. aureus	0.01 ± 0.01[a]	0.01 ± 0.01[a]	0.05 ± 0.01[a]	0.27 ± 0.03[a]	0.86 ± 0.06[a]
	Significance	*	*	*	*	*
Acetic acid	*E. faecalis*	0.13 ± 0.01[b]	0.36 ± 0.03[b]	0.50 ± 0.02[b]	0.57 ± 0.00[c]	0.61 ± 0.00[c]
	Co-culture	0.14 ± 0.01[b]	0.36 ± 0.01[b]	0.49 ± 0.01[b]	0.55 ± 0.00[b]	0.58 ± 0.00[b]
	S. aureus	0.09 ± 0.00[a]	0.09 ± 0.01[a]	0.11 ± 0.01[a]	0.27 ± 0.00[a]	0.35 ± 0.00[a]
	Significance	*	*	*	*	*

[a,b,c]For each metabolite, different letters in the same column indicate difference at 95% level of significance; ns: not significant; nd: not detected.

fermented foods, can be a major aspect in limiting *S. aureus* growth (Noleto et al., 1987; Smith et al., 1983).

SE production has been reported to be dependent upon cell density ($\geq 10^6$ CFU/mL or g) (Schelin et al., 2011), as well as affected by the medium (Charlier et al., 2008; Noleto et al., 1987), environmental cues, carbon sources and competing microbiota (Smith et al., 1983). In our co-culture assays, SEs were absent in cheeses where population reached maximum levels of 10^5 CFU/g (Table 2), but SEs were present in BHI

Table 5
pH and metabolite concentration (g/Kg) in miniaturised soft fresh cheeses inoculated with *E. faecalis* 41FL1 and *S. aureus* ATCC 29213 alone and in combination during 21 days of storage at 15 °C.

Chemical parameter	Assay	Days				
		1	3	7	15	21
pH	*E. faecalis*	4.98 ± 0.00[a]	4.88 ± 0.00[a]	4.80 ± 0.00[a]	4.77 ± 0.01[a]	4.71 ± 0.00[b]
	Co-culture	4.99 ± 0.00[a]	4.88 ± 0.00[a]	4.81 ± 0.00[b]	4.79 ± 0.01[a]	4.70 ± 0.00[a]
	S. aureus	6.74 ± 0.05[b]	6.21 ± 0.08[b]	5.20 ± 0.00[c]	5.01 ± 0.01[b]	4.96 ± 0.02[c]
	Significance	*	*	*	*	*
Lactose	*E. faecalis*	24.58 ± 3.62[a]	17.85 ± 3.22[a]	10.76 ± 4.38[a]	9.99 ± 0.68[a]	10.12 ± 1.00[a]
	Co-culture	25.40 ± 0.64[a]	18.33 ± 1.50[a]	10.74 ± 3.64[a]	10.31 ± 0.64[a]	10.24 ± 0.67[a]
	S. aureus	42.13 ± 0.59[b]	39.19 ± 4.57[b]	27.93 ± 1.17[b]	19.79 ± 1.90[b]	16.20 ± 2.87[b]
	Significance	*	*	*	*	*
Galactose	*E. faecalis*	0.31 ± 0.05[b]	0.49 ± 0.12[b]	1.05 ± 0.39[b]	1.21 ± 0.17[b]	0.98 ± 0.21[c]
	Co-culture	0.25 ± 0.01[b]	0.38 ± 0.11[b]	0.89 ± 0.35[b]	1.00 ± 0.24[b]	0.66 ± 0.08[b]
	S. aureus	0.11 ± 0.00[a]	0.09 ± 0.07[a]	0.08 ± 0.05[a]	0.05 ± 0.01[a]	0.12 ± 0.08[a]
	Significance	*	*	*	*	*
Citric acid	*E. faecalis*	nd[a]	nd[a]	nd[a]	0.16 ± 0.01[b]	0.09 ± 0.06[b]
	Co-culture	nd[a]	nd[a]	nd[a]	nd[a]	nd[a]
	S. aureus	1.04 ± 0.06[b]	1.19 ± 0.03[b]	0.87 ± 0.13[b]	0.91 ± 0.06[c]	0.60 ± 0.06[c]
	Significance	*	*	*	*	*
Pyruvic acid	*E. faecalis*	0.08 ± 0.00[c]	0.08 ± 0.00[c]	0.12 ± 0.03	0.19 ± 0.02	0.20 ± 0.03
	Co-culture	0.06 ± 0.00[b]	0.06 ± 0.00[b]	0.17 ± 0.02	0.18 ± 0.01	0.18 ± 0.01
	S. aureus	0.01 ± 0.00[a]	0.03 ± 0.02[a]	0.15 ± 0.02	0.15 ± 0.03	0.24 ± 0.05
	Significance	*	*	ns	ns	ns
Lactic acid	*E. faecalis*	19.99 ± 1.22[c]	26.88 ± 2.72[c]	34.68 ± 4.26[b]	38.09 ± 3.37[c]	37.40 ± 3.05[c]
	Co-culture	15.72 ± 0.09[b]	22.11 ± 1.56[b]	28.56 ± 2.88[b]	31.14 ± 0.80[b]	31.61 ± 2.61[b]
	S. aureus	0.03 ± 0.01[a]	0.33 ± 0.31[a]	11.69 ± 3.02[a]	13.23 ± 1.88[a]	19.88 ± 4.89[a]
	Significance	*	*	*	*	*
Acetic acid	*E. faecalis*	1.57 ± 0.14[c]	1.43 ± 0.09[c]	1.57 ± 0.18[b]	1.81 ± 0.32[a]	1.77 ± 0.47[a]
	Co-culture	1.20 ± 0.14[b]	1.13 ± 0.15[b]	1.20 ± 0.12[a]	1.33 ± 0.19[a]	1.48 ± 0.05[a]
	S. aureus	0.07 ± 0.00[a]	0.15 ± 0.01[a]	1.09 ± 0.20[a]	3.57 ± 0.35[b]	3.58 ± 0.10[b]
	Significance	*	*	*	*	*
Acetoin	*E. faecalis*	0.49 ± 0.13[b]	1.05 ± 0.32[b]	1.21 ± 0.14[b]	1.84 ± 0.16[b]	1.94 ± 0.16[b]
	Co-culture	0.45 ± 0.22[b]	1.95 ± 0.25[c]	2.12 ± 0.20[c]	2.24 ± 0.21[c]	2.88 ± 0.27[c]
	S. aureus	nd[a]	nd[a]	0.51 ± 0.04[a]	0.74 ± 0.15[a]	1.02 ± 0.59[a]
	Significance	*	*	*	*	*

For each metabolite, different letters in the same column indicate difference at 95% level of significance; ns: not significant; nd: not detected.

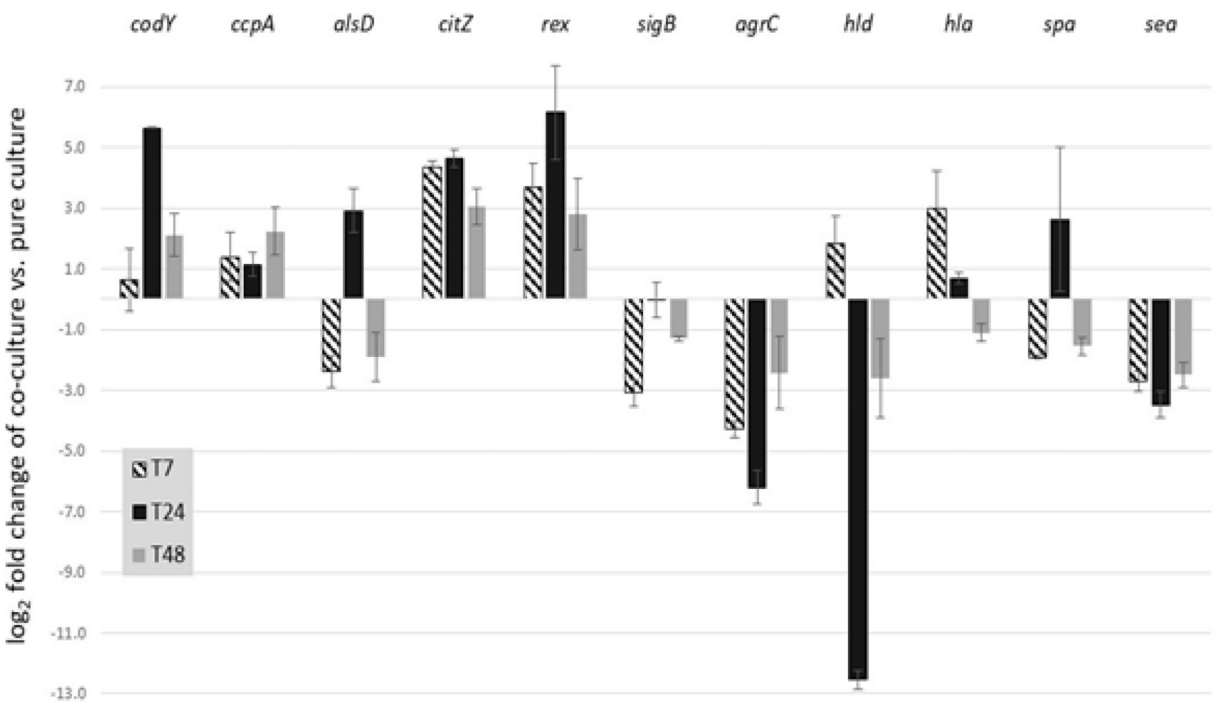

Fig. 1. Expression pattern of *S. aureus* selected genes in BHI broth as determined by qRT-PCR. Gene expressions are reported relative to *mqo2* and calculated using the $2_T^{-\Delta\Delta C}$ method.

after 48 h of incubation when contamination of *S. aureus* reached 10^6 CFU/mL (Table 3). Noleto et al. (1987) found that *S. aureus* strains were able to produce SEs in mixed culture with *E. faecalis* at 37 °C in BHI broth but not in meat slurry at initial inoculum sizes ranging from 10 to 10^3 CFU/mL. Additional studies focusing on cheese models reported the absence of SEs with *S. aureus* at 10^3 CFU/g as initial inoculum (Delbes et al., 2006; Gómez-Lucía et al., 1992; Meyrand et al., 1998). Thus, in co-cultured BHI, the occurrence of SEs can be attributed, at least partly, to high population density; however, non-detection of SEs in our co-cultured cheese model might be related to an insufficient number of *S. aureus* cells coupled with environmental conditions determined by the surrounding matrix, such as low pH, that might be affecting full SE expression.

pH might have partially hindered *S. aureus* growth and enterotoxigenic ability in co-cultivated matrices due to acidification resulted from *E. faecalis* activity, as demonstrated by the increasing levels of lactate in both studies matrices (Tables 2 and 3). Previous studies agree that the repressive effect of LAB over *S. aureus* due to lactate production might potentially indicate *S. aureus* growth and survival in foods, if low pH values are reached within the first 6 h of concomitant

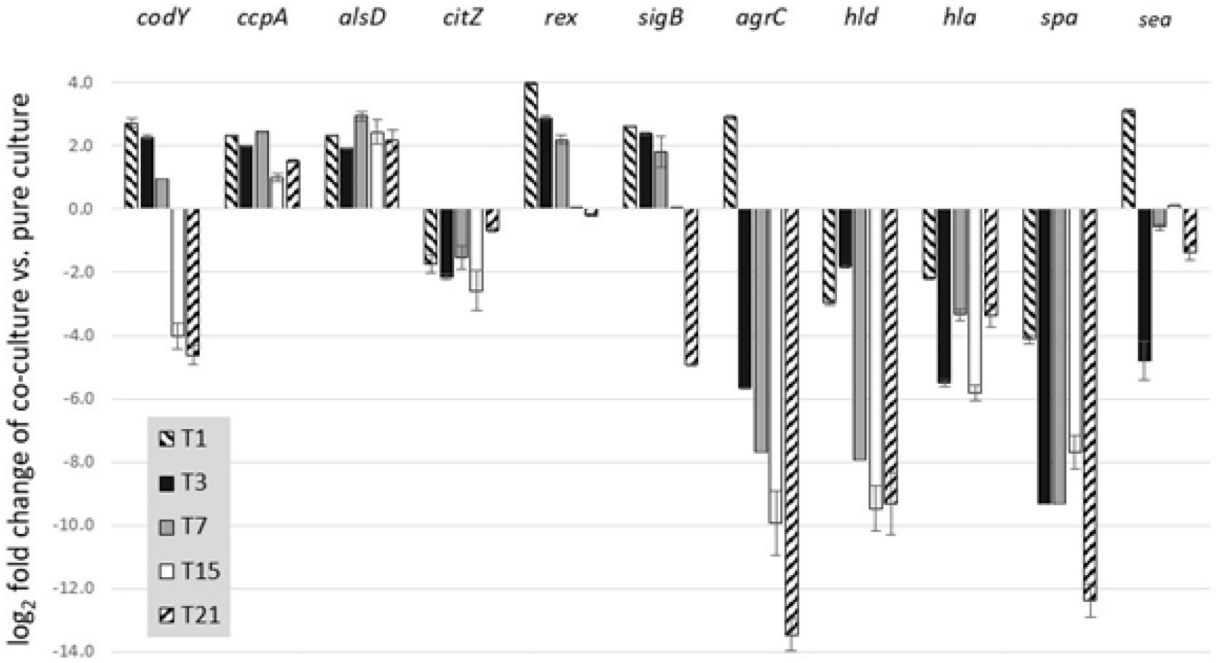

Fig. 2. Expression pattern of *S. aureus* selected genes in miniaturised soft fresh cheese as determined by qRT-PCR. Gene expressions are reported relative to *mqo2* and calculated using the $2_T^{-\Delta\Delta C}$ method.

growth (Delbes et al., 2006). Conversely, it has also been suggested that acidification by LAB exert only a minor role in repressing *S. aureus*, pointing towards cell-to-cell or nutrient-related phenomena (Charlier et al., 2008; Haines and Harmon, 1973). In our study, pH-related phenomena were more likely to have led to early arrest of *S. aureus* growth in co-cultured matrices, as well as negatively impacted SE production in co-inoculated cheeses where pH reached values below 5.0, considered to be the least permissive pH value for SE production (Table 5).

In the last decade, significant amount of research has provided insights on the interaction between *S. aureus* and LAB in controlled conditions through the use of molecular or "omic" tools (Alomar et al., 2008; Charlier et al., 2008; Cretenet et al., 2011; Delpech et al., 2015; Even et al., 2009; Viçosa et al., 2018). These studies have shown that despite a moderate negative impact on growth, *S. aureus* gene expression is strongly affected by LAB, particularly the expression of virulence related genes. In the present study, expression of *agrC* and *hld*, which are key genes of the global virulence regulator *agr* system in *S. aureus* (Novick, 2003), was reduced in co-inoculated matrices (Figs. 1 and 2). Overall, our results are in line with a prevalent concept that mechanisms by which LAB repress *S. aureus* are not species-specific but rather linked to properties commonly shared by LAB (Charlier et al., 2008).

Growth of *S. aureus* in the presence of LAB is likely to be accompanied by several types of stresses due to acidification, competition for nutrients, increased proteolysis and decreased oxygen availability. *S. aureus* has evolved several strategies to couple responses to signals derived from environmental stimuli with modulation of metabolic status and virulence (Rode et al., 2012). Among these strategies, the role of transcriptional regulators, such as *sigB*, *rex, codY and ccpA*, has been widely studied (Pagels et al., 2010; Seidl et al., 2006; Tuchscherr et al., 2015; Waters et al., 2016). *sigB* expression occurs under environmental stressing conditions (e.g. acidic pH, low redox potential) and is linked to attenuated virulence and reduced metabolism in *S. aureus* (Tuchscherr et al., 2015). *rex* has a direct regulatory effect on the expression of metabolic pathways that mediate NAD+ regeneration and ATP synthesis in *S. aureus*, such as formation of lactate, ethanol, and formate (Pagels et al., 2010). *codY* and *ccpA* are metabolic and nutrient-responsive regulators that link *S. aureus* metabolic status to the transcriptional regulation of several virulence factors and the *agr* system (Majerczyk et al., 2008; Seidl et al., 2006). We demonstrated here that the expression of such regulators in *S. aureus* was mostly upregulated in BHI and cheese in the presence of *E. faecalis* (Figs. 1 and 2). The upregulation of *codY* and *ccpA* in *S. aureus* has been associated with attenuated virulence in single culture in laboratory media (Majerczyk et al., 2008; Seidl et al., 2006), as well as in co-culture with *Lactococcus lactis* in a cheese-like matrix (Cretenet et al., 2011) and with *E. faecalis* in skimmed milk (Viçosa et al., 2018), which helps explain our findings.

The reducing potential of LAB, i.e. their ability to consume oxygen from the environment (Brasca et al., 2007), has been proposed to negatively impact *S. aureus* virulence, in particular the expression of the *agr* system (Nouaille et al., 2014). In *S. aureus*, the transcriptional regulator Rex senses intracellular changes in the NADH/NAD^+ ratio and upregulates the expression of genes linked to anaerobic NAD^+ regeneration, such as glycolysis and fermentation pathways (Pagels et al., 2010). Under oxygen-limiting conditions, which might be the case of our co-culture conditions, excessive NADH production might be avoided by decreasing the synthesis of components of the tricarboxylic acid (TCA) cycle, which likely leads to the accumulation of end products of fermentation, such as lactate, acetate, and acetoin (Fuchs et al., 2007; Pagels et al., 2010). In particular, the role of acetoin in counteracting the deleterious effects of acidic stress in *S. aureus* by alkalinisation of the cytoplasm and enhancing survival has been previously described (Thomas et al., 2014). In our study, *rex* was found to be constantly upregulated in matrices where *S. aureus* was co-inoculated with *E. faecalis* (Figs. 1 and 2). Transcription of *citZ*, encoding the enzyme citrate synthase that catalyses the first reaction of the TCA cycle to generate citrate, was downregulated in *S. aureus* in co-cultured cheese. Interestingly, citrate was present only in cheese containing *S. aureus* alone, as a result of the TCA cycle activity in such conditions. Conversely, acetate was found in reduced amounts in co-inoculated cheese, but accumulated significantly in cheese with *S. aureus* alone. In addition, concentration of acetoin was significantly higher in co-inoculated cheese, an evidence corroborated also by an increased expression of *alsD* in this matrix, which encodes the enzyme that converts acetolactate into acetoin. These findings can thus be regarded as an adaptation strategy of *S. aureus* to withstand lowered redox potential and pH as the result of the activity of *E. faecalis* in the cheese matrix by diverging carbon flux towards more neutral metabolic byproducts (acetoin) rather than forming acetate, which is known to trigger cell death in *S. aureus* under low pH (Thomas et al., 2014).

The ability of certain *S. aureus* strains to produce several SE is the foremost concern in foodstuffs. SEA, a prophage-encoded *S. aureus* enterotoxin (Betley et al., 1984), is among the most frequently associated with SFP (Pinchuk et al., 2010). *sea* expression occurs between exponential and stationary growth phase and is not regulated by the *agr* locus (Tremaine et al., 1993). Previous work has described the influence of pH on *sea* expression, in which no *sea* mRNA was detected at pH below 5.0 in liquid medium acidified with acetate (Wallin-Carlquist et al., 2010). In our study, *sea* transcripts of *S. aureus* ATCC 29213 were downregulated in both co-cultured matrices (Figs. 1 and 2); SEA was present in co-cultured BHI at 48 h of incubation at 30 °C (Table 2) but not in co-cultured cheese (Table 3). Thus, pH decrease induced by the presence of *E. faecalis* in the cheese matrix could explain the failure of *S. aureus* to produce SEA in co-inoculated cheese, while pH values reached in co-cultured BHI might have still been permissive to the production of SEA (Wallin-Carlquist et al., 2010).

In summary, our data indicate that *S. aureus* might modulate its metabolism during growth with *E. faecalis* in BHI and soft cheeses to enhance survival, while compromising its ability to produce SE in an acidic and reduced environment such as the cheese-like matrix. Our data add up to the current body of knowledge on how *S. aureus* copes with the presence of positive microbiota in lab and food-like media and contribute towards a better understanding of how using controlled population dominance by LAB can be an effective biopreservation strategy for ensuring food safety.

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Declaration of competing interest

None.

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