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Staphylococcus aureus undergoes major transcriptional reorganization during growth with *Enterococcus faecalis* in milk

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Abstract

Previous studies have demonstrated the antagonistic potential of lactic acid bacteria (LAB) present in raw milk microbiota over *Staphylococcus aureus*, albeit the molecular mechanisms underlying this inhibitory effect are not fully understood. In this study, we compared the behavior of *S. aureus* ATCC 29213 alone and in the presence of a cheese-isolated LAB strain, *Enterococcus faecalis* 41FL1 in skimmed milk at 30 °C for 24 h using phenotypical and molecular approaches. Phenotypic analysis showed the absence of classical staphylococcal enterotoxins in co-culture with a 1.2-log decrease in *S. aureus* final population compared to single culture. Transcriptional activity of several exotoxins and global regulators, including *agr*, was negatively impacted in co-culture, contrasting with the accumulation of transcripts coding for surface proteins. After 24 h, the number of transcripts coding for several metabolite responsive elements, as well as enzymes involved in glycolysis and acetoin metabolism was increased in co-culture. The present study discusses the complexity of the transcriptomic mechanisms possibly leading to *S. aureus* attenuated virulence in the presence of *E. faecalis* and provides insights into this interspecies interaction in a simulated food context.

Keywords: *Staphylococcus aureus*; *Enterococcus faecalis*; RNA sequencing; co-culture; skimmed milk

1. Introduction

Staphylococcus aureus is a pathogen of major concern in foodstuffs due to the ability of certain strains to produce thermoresistant staphylococcal enterotoxins (SE) that, once ingested, may lead to gastrointestinal disorders symptoms, such as vomiting, nausea and abdominal cramping (Argudín et al., 2010; Schelin et al., 2011). Despite remarkable improvements in food safety procedures, SE still figure as a leading cause of foodborne outbreaks in Europe, often related to the ingestion of contaminated milk and dairy products (EFSA, 2016).

Lactic acid bacteria (LAB) are heavily present in raw milk microbiota and have been extensively used in the food industry for the production of dairy and non-dairy fermented products (Stiles and Holzapfel, 1997). In this context, LAB can exert a protective role against the multiplication of spoilage and pathogenic microorganisms, mainly by modulating redox potential, pH or through the production of inhibitory substances (Carr et al., 2002). Among LAB, *Enterococcus faecalis* is one of the most frequent species recovered from milk and cheese in the Mediterranean area, reaching levels of 10^5 to 10^7 CFU/g at the end of cheese ripening and contributing to the development of organoleptic characteristics (Foulquié-Moreno et al., 2006; Franciosi et al., 2009).

The conditions supporting *S. aureus* growth and enterotoxin production in foods are extensive, since *S. aureus* is able to grow and survive in a wide range of temperatures, pH and NaCl concentrations (Valero et al., 2009). The expression of several virulence determinants in *S. aureus* is tightly coordinated to environmental conditions by a complex gene regulatory network, which is mainly controlled by the accessory gene regulator (*agr*) system (Novick et al., 1993). By responding to stress and external factors, *S. aureus* can rapidly adapt its physiology and virulence in order to optimize growth and survival in complex environments, such as foods (Novick et al., 1993).

Although LAB antagonistic potential over *S. aureus* in foods has been the subject of research for more than fifty years, most of these early observations relied mainly on

phenotypical analysis to study this interaction (Daoud and Debevere, 1985; Haines and Harmon, 1973; Hamama et al., 2002; Noleto et al., 1987; Perin et al., 2012). In recent years, application of molecular techniques has proven to be a reliable strategy to explore the LAB-*S. aureus* interaction in culture and food-mimicking media (Charlier et al., 2008; Cretenet et al., 2011; Delpech et al., 2015; Even et al., 2009; Zdenkova et al., 2016). However, only a few studies have been able to successfully determine the underlying mechanism leading to attenuated *S. aureus* virulence in mixed bacterial cultures (Laughton et al., 2006; Li et al., 2011).

In the present study, we monitored the behavior of *S. aureus* alone and in the presence of *E. faecalis* during growth in skimmed milk. We used RNA sequencing analysis to explore the adaptations in the *S. aureus* transcriptome that could, at least partly, support our phenotypical findings, i.e., the reduction in *S. aureus* final population and the absence of SEs in co-culture with *E. faecalis*. To the best of our knowledge, this study is the first transcriptomic approach of the interaction between *S. aureus* and *E. faecalis* in skimmed milk and provides interesting knowledge to for designing biocontrol-based strategies to improve food safety.

2. Material and methods

2.1. Bacterial strains and growth conditions

S. aureus ATCC 29213 and *E. faecalis* 41FL1 (Dal Bello et al., 2010) were used in this work. To evaluate bacterial interactions, *S. aureus* was inoculated alone and in combination with *E. faecalis* in 100 mL of skimmed milk (Sigma-Aldrich, St. Louis, USA) in 250-mL Schott® flasks and statically incubated at 30° C for 24 h. Skimmed milk powder was reconstituted in sterile distilled water (10% w/v) in aseptic conditions followed by treatment at 121 °C for 5 min before bacterial inoculation. Both strains were subcultured at 30° C for 18 h and inoculated at a final concentration of 10^3 CFU/mL for *S. aureus* and 10^6 CFU/mL for *E. faecalis*. Experiments were repeated three times to ensure reproducibility.

99

100 *2.2. Microbiological analysis*

101 Throughout the incubation period, bacterial growth was assessed at the following
102 intervals: 0 h (inoculation); 4 and 7 h (exponential phase); 12 and 24 h (post-exponential
103 phase). CFU of *S. aureus* population in single and co-cultures was determined on Baird-
104 Parker Rabbit Plasma Fibrinogen agar (bioMérieux, Marcy-l'Étoile, France) (De Buyser et
105 al., 2003), whereas *E. faecalis* population was enumerated on BHI agar (Sigma-Aldrich)
106 followed by a confirmatory step with the catalase test. All plates were incubated at 37° C for
107 48 h. Bacterial growth was determined in duplicate using the spread plate technique for each
108 sampling point.

109

110 *2.3. Enterotoxin detection*

111 At the same intervals set for *S. aureus* enumeration, samples for SE detection were
112 collected and immediately analyzed. SE production was qualitatively estimated in samples
113 using RIDASCREEN® SET TOTAL (R-Biopharm AG, Darmstadt, HE, GE) according to
114 manufacturer's instructions.

115

116 *2.4. Determination of pH, sugars and organic acids content*

117 Sugars and organic acids contents in milk were determined by high performance
118 liquid chromatography (HPLC) using the method described by Bertolino et al. (2011) with
119 minor modifications. Briefly, 5 mL of milk samples were added to 20 mL of 0.013 N H_2SO_4
120 (mobile phase) and mixed for 30 min with a horizontal shaker (Asal, Milan, Italy) at 100
121 oscillation/min. The samples were subsequently centrifuged for 5 min at 10000 X *g* and 10
122 °C, and the supernatant was filtered through a 0.2 µm polypropylene membrane filter (VWR,
123 Milan, Italy). The HPLC system (Thermo Finnigan Spectra System, San Jose, USA) was
124 equipped with an isocratic pump (P4000), a multiple autosampler (AS3000) fitted with a 20
125 µL loop, a UV detector (UV100) set at 210 and a refractive index detector RI-150. The

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 analyzed for each biological replicate. The data treatments were carried out using the Chrom QuestTM chromatography data system (Thermo Finnigan Spectra System, San Jose, USA). Analytical grade reagents were used as standards (Sigma-Aldrich). pH measurements were obtained using a pH meter at the same intervals considered in the HPLC analysis (Crison, Modena, Italy).

2.5. Whole genome sequencing

Genomic DNA (gDNA) was extracted from a *S. aureus* ATCC 29213 culture in BHI broth grown to stationary phase using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to manufacturer's instructions. Whole genome sequencing (WGS) was performed at GenProbio srl (Parma, Italy) using the Illumina MiSeq platform (Illumina, San Diego, CA, USA). From 2.5 µg of gDNA, a library for Illumina paired-ended sequencing was prepared using the TruSeq DNA PCR-Free LT Kit (Illumina), combined with fragmentation using a Bioruptor NGS ultrasonicator (Diagenode, LI, Belgium) and size evaluation using Tape Station 2200 (Agilent Technologies, Palo Alto, CA, USA). The library was sequenced (2 x 250 bp) using a Flow Cell V3 600 cycles (Illumina) according to the manufacturer's instructions. *De novo* genome assembly was performed with MIRA v.4.0.2 (Chevreux et al., 1999). Improvement quality of final contigs was performed with Burrows-Wheeler Aligner (Li and Durbin, 2009), SAMtools suite (Li et al., 2009) and GATK software package v.2.8-1 (McKenna et al., 2010). Reordering of the final contigs was performed with Mauve v.2.3.1 (Darling et al., 2004) against *S. aureus* NCTC 8325 as reference genome (NCBI Accession Number: NC_007795.1). Ribosomal RNA genes were detected on the basis of RNAmmer v.1.2 (Lagesen et al., 2007) and transfer RNA genes were identified using tRNAscan-SE v.1.21 (Lowe and Eddy, 1997). Protein-encoding open reading

153 frames (ORFs) were predicted using Prodigal v.2.6 (Hyatt et al., 2010). Automatic annotation
154 of the ORFs was performed with BLAST (Altschul et al., 1990) against NCBI database and
155 HMMER against the PFAM database (Sonnhammer et al., 1997). Manual corrections to
156 automated functional assignments were conducted to validate the presence or absence of
157 genes of interest. Based on the annotation, coding DNA sequences (CDS) were classified into
158 the Cluster of Orthologous Groups (COG) functional categories (Tatusov et al., 2000) using
159 EggNog v.4.0 database (Jensen et al., 2008).
160
161 *2.6. Transcriptomic analysis by RNA-Seq and gene expression analysis*
162 Bacterial cells from single and co-cultures in duplicates were harvested after 7 and 24
163 h of incubation at 30 °C, and total RNA was isolated using the RiboPure™ Bacteria kit
164 (Ambion, Life Technologies, Waltham MA). RNA was treated with DNase I (Turbo DNA
165 free; Ambion) to ensure complete removal of gDNA. RNA quantification and integrity were
166 determined by agarose gel electrophoresis and Agilent 2200 Tape Station Nucleic Acid
167 System (Agilent Technologies). Following quantification, rRNA was removed from 50 ng of
168 total RNA using Ribo-Zero rRNA removal kit for Gram-positive bacteria (Epicentre,
169 Madison, WI, USA) according to the supplier's instructions. The yield of rRNA depletion
170 was checked by Agilent 2200 Bioanalyzer (Agilent Technologies). rRNA-depleted samples
171 were then fragmented using RNaseIII (Life Technologies, CA, USA) followed by size
172 evaluation using Experion (Bio-Rad, CA, USA). RNA-Seq library was constructed with the
173 IonTorrent Total-RNAseq kit v2 (Life Technologies) according to the manufacturer's
174 protocol. Library templates were amplified on Ion Sphere Particles using Ion One Touch 200
175 Template Kit v2 (Life Technologies). Sequencing of libraries was loaded into IonTorrent 316
176 chips using an Ion PGM 200 sequencing kit (Life Technologies) at GenProbio srl (Parma,
177 Italy). Sequencing reads of each replicate were pooled and aligned to the *S. aureus* ATCC
178 29213 genome sequence through BWA39 with high stringency cut-offs (99% nucleotide
179 identity) to accurately assign co-culture reads to the correct genome. Alignment data were

analyzed using HTSeq (http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html). Raw mapped reads counts of each gene were normalized to the length of the gene itself and to the number of reads mapped. The resulting data is presented as reads per kilobase of transcript per million reads mapped (RPKM) and allows the comparison of genes in different samples at different depths of sequencing. Based on RPKM values, we determined transcriptional changes between *S. aureus* in single and co-culture by fold change analysis, using the single culture as a reference. Genes were considered differentially expressed when $\log_2$ fold change (LFC) was $\geq +1$ or ≤ -1.

2.7. Real-time quantitative reverse transcription PCR (RT-qPCR)

Six *S. aureus* genes (*codY*, *alsD*, *agrC*, *spa*, *hld*, *sea*) up- or downregulated by more than 2-fold at 7 h or 24 h in co-culture were selected to validate the data generated from RNA-Seq experiments by RT-qPCR. *mqo2* was used as reference gene for RT-qPCR data normalization since its expression profile remained invariant in all transcriptomes. The target genes were selected based on their role as virulence factors and in central carbon metabolism. The primer pairs used in RT-qPCR and reverse transcription reactions were designed using the Primer-BLAST tool (Ye et al., 2012) based on the genome sequence of *S. aureus* ATCC 29213. Further confirmation of *in silico* specificity of selected primer sequences was performed with UGENE software version 1.26.1 (Okonechnikov et al., 2012) and BLAST (Altschul et al., 1990) against NCBI database. All primer sequences used in this study are listed in Supplementary Table S5. Gene-specific reverse transcription was conducted as follows: 600 ng of RNA were mixed with 1 µL of reverse primer (100 µM) and ultrapure water in a reaction volume of 10 µl. The mix was treated at 75 °C for 5 min for RNA denaturation and immediately placed on ice for 10 min. Five microliters of M-MLV RT Buffer (1 X), 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) with subsequent storage of cDNA at -20 °C. RT-qPCR reactions were performed on corresponding cDNAs in a final volume of 20 µL using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) as recommended by the manufacturer in a MJ Research PTC-200 DNA Engine® Peltier Thermal Cycler (Bio-Rad) with the following cycle conditions: initial denaturation at 98 °C for 30 sec, followed by 40 cycles of 95 °C for 15 sec and a 30-second step at 59.4 °C for *codY* and *alsD*, 54.8 °C for *agrC* and *spa*, 51.4 °C for *hld* and *sea*. The specificity of primer sets used for qPCR amplification was evaluated by melting curve analysis. All reactions were independently conducted three times on two biological replicates. Gene expression data analysis using the $2^{-\Delta\Delta C_T}$ method, correlation coefficients of oligonucleotides and efficiency of amplifications were calculated as described previously (Livak and Schmittgen, 2001).

2.8. Sequencing data access

The annotated genome was submitted to the National Center for Biotechnology Information (NCBI) database (Bioproject accession number: PRJNA344949). RNA-Seq data generated in this experiment was deposited in the Sequence Read Archive (Leinonen et al., 2011) of the NCBI and is publicly available through the accession number SRP092596.

2.9. Statistical analysis

Data from HPLC analysis were subjected to one-way analysis of variance (ANOVA), while differences in mean counts of microbial populations in single and co-culture experiments were assessed by a Student's t-test using Statistica software version 6 (Statsoft, Tulsa, OK, USA). Statistical significance was set at $p < 0.05$.

3. Results

3.1.Growth kinetics, enterotoxin production and milk acidification

The mean counts of *S. aureus* ATCC 29213 and *E. faecalis* 41FL1 populations in single and co-culture, as well as SE production, in sterile skimmed milk at 30 °C for 24 h are reported in Table 1. pH measurements and water-soluble metabolites present in milk of single and co-culture are reported in Table 2. *S. aureus* growth was hindered in co-culture with *E. faecalis* compared to single culture, resulting in a 1.2-log inhibition after 24 h. The ability of *S. aureus* to produce enterotoxins was verified only at 24 h in single culture, whereas no SE was detected in co-culture throughout the incubation period (Table 1). Interestingly, *S. aureus* ATCC 29213 showed virtually no impact on the growth dynamic of *E. faecalis* 41FL1, as the latter largely followed the same growth pattern whether in single or co-culture, reaching a final population density of approximately 1.0 x 10^9 CFU/mL after 24 h. Progressive acidification of milk was observed in the presence of *E. faecalis* in single and co-culture, with final pH values lower than 5 simultaneously with an increasing production of lactic acid and consequently decrease in lactose content in milk (Table 2). Conversely, pH values, lactic acid, and lactose contents remained virtually unaltered in *S. aureus* single culture.

3.2. S. aureus *ATCC 29213 genome sequencing and transcriptomic response*

The complete sequencing of *S. aureus* ATCC 29213 genome was obtained by Illumina MiSeq Sequencing System technology for 250-bp paired-end sequencing. A total of 86 contigs were assembled using MIRA version 4.0.2 to provide a genome length of 2,847,591 bp with an average GC content of 32.81%. Sequencing of *S. aureus* ATCC 29213 genome revealed the presence of 2,676 CDS and 120 RNAs (19 rRNAs, 99 tRNAs and 2 pseudogenes). The number of sequences obtained in the RNA-Seq experiments ranged from 224,614 to 804,266 for each replicate, with a total of 4,017,675 read counts. For gene expression studies, the transcriptome of *S. aureus* in single culture was used as a reference for assessing changes in gene expression in co-culture by means of fold change analysis. Therefore, we refer to upregulated and downregulated in comparing transcript levels between

the two conditions. Data was analyzed using an arbitrary cutoff value of LFC between -1 and +1.

In Figure 1, the transcriptomic response of *S. aureus* ATCC 29213 in single and co-culture was divided into 5 categories depicting the frequency of genes belonging to each pattern over time. After 7 h of incubation, 303 genes were differentially expressed in co-culture, of which 257 genes were upregulated and 46 genes were downregulated. After 24 h, *S. aureus* transcriptome in co-culture showed an increased number of transcripts belonging to 597 genes and a reduced number of transcripts coding for 63 genes. In both time intervals, 9 genes were found to be transcribed in lower rates whereas transcripts of 59 genes were significantly enriched in co-culture (Figure 1A and 1B; Tables S1 and S2). Considering single and co-culture transcriptomes, transcripts coding for 748 genes were absent at 7 h, whereas this number decreased to 197 genes at 24 h, with 146 shared genes (Figure 1C). Moreover, transcripts coding for 1,375 and 1,512 genes was not detected in co-culture at 7 and 24 h, respectively, with 856 overlapping genes in this pattern (Figure 1D). Finally, transcription of 35 and 39 genes occurred exclusively in co-culture at 7 and 24 h, respectively, sharing no common representatives (Figure 1E; Table S3 e S4). Overall, these observations indicate that the global transcriptomic response of *S. aureus* in co-culture with *E. faecalis* diverges considerably from that observed in single culture.

To validate the RNA-Seq experiments, the expression of six *S. aureus* genes (*codY*, *alsD*, *agrC*, *spa*, *hld*, *sea*) up- or downregulated in co-culture by more than twofold was evaluated by RT-qPCR. Overall, trends in the expression of selected genes as determined by RT-qPCR corroborated the RNA-Seq findings, with minor inconsistencies (Figure S1). Notably, RT-qPCR showed an upregulation of *codY* by 6-fold in co-culture at 7 h, whereas *alsD* showed a converse expression pattern from that obtained with RNA-Seq. The expression of *agrC*, *spa*, *hld* and *sea* was found to be strongly decreased in co-culture at 7 h and 24 h according to RT-qPCR analysis.

Gene expression analysis based on the relative abundance of transcripts assigned to their respective COG categories revealed the occurrence of distinct transcriptional response profiles in co-culture in each tested interval (Figure 2). To guide our analysis, we calculated the relative percentage difference of total RPKM values obtained in each COG category at 7 and 24 h in co-culture versus single culture, and arbitrarily chosen to focus on COG categories with changes of plus or minus 40%. After 7 h of incubation, a large amount of transcripts in co-culture were assigned to categories 'Transcription' (+44.51%), 'Replication, recombination and repair' (+182.94%) and 'Posttranslational modification, protein turnover, and chaperones' (+72.81%), while the expression of genes related to the 'Nucleotide transport and metabolism' (-45.93%) was negatively impacted, which strongly indicates the triggering of stress response cascades in co-culture (Figure 2A). In contrast, after 24 hours of incubation, the categories 'Carbohydrate transport and metabolism' (+108.78%), Coenzymes transport and metabolism (+60.59%) and 'Secondary metabolites biosynthesis, transport and catabolism' (+46.02%) were expressively more abundant in co-culture, whereas transcripts encoding proteins assigned to the category 'Translation, ribosomal structure, and biogenesis' had considerably decreased expression compared to single culture (-45.23%), suggesting major adaptations in *S aureus* central metabolism (Figure 2B).

Compared to single culture, the presence of transcripts encoding enzymes involved in the glycolytic and tricarboxylic acid (TCA) cycle pathways was lower in co-culture at 7 h (Figure 3). In particular, transcription of genes coding for six important glycolytic enzymes (*fbaA*, *fda*, *tpiA*, *gapA1*, *pgm* and *pykA*) was 4.2, 2.0, 7.9, 2.7, 2.6 and 2.8-fold lower in co-culture at 7 h compared to single culture, respectively. Transcripts encoding enzymes of the pyruvate dehydrogenase complex (*pdhA*, *pdhB*, *pdhC*, *pdhD*) and citrate synthase (*citZ*) did not accumulate in co-culture to the same extent as in single culture at 7 h. However, after 24 hours of incubation, we observed a noteworthy enrichment in the transcripts coding for enzymes involved in glycolysis, acetoin and acetate metabolism (Figure 3). Transcripts of genes coding for all glycolytic enzymes, except for *gapA1*, accumulated significantly in co-

culture compared to single culture at 24 h. Likewise, transcripts of acetolactate synthase (*alsS*), acetolactate decarboxylase (*alsD*) and acetoin reductase (*butA*) involved in the 2,3-butanediol pathway and acetoin production were present in significantly larger amounts in co-culture at 24 h compared to single culture.

Considering the transcription of genes belonging to the *agr* locus (*agrBCDA*) in *S. aureus* single and co-culture, we noted a transient upregulation of *agrC* by 10-fold in co-culture at 7 h, but no transcripts of the *agrBDCA* locus were detected in co-culture at 24 h. The RNAIII transcript encoding the δ-hemolysin (Hld) was not detected in the co-culture transcriptome at neither 7 h nor 24 h, while high amounts of this transcript were found in single culture at 24 h accompanied by the presence of transcripts of *agrBCDA* (Table 3). In addition, transcripts of other notable global transcription regulators of *S. aureus* virulence, such as *mgrA* (Crosby et al., 2016), *saeS*, *saeP* (Giraudo et al., 1997), and several *sarA* homologs (Cheung et al., 2004) were absent in co-culture at 7 h or 24 h (Table 3). Accordingly, transcripts coding for several *S. aureus* secreted toxins and exoenzymes were absent or present in significantly reduced numbers in co-culture at 24 h, whereas transcripts coding for several cell surface-associated proteins were upregulated in the same conditions (Table 3).

4. Discussion

A deeper knowledge concerning bacterial interactions is needed to support new ways of thinking the control of growth and virulence of pathogenic microorganisms in food products. Although considerable research has been dedicated to investigating the negative impact of certain LAB strains over the ability of *S. aureus* to grow and produce enterotoxins in food-mimicking conditions (Alomar et al., 2008; Daoud and Debevere, 1985; Kao and Frazier, 1966; Noleto et al., 1987), rather less amount of knowledge has been pulled together concerning the molecular mechanisms underlying this antagonistic phenomenon. In the present study, we demonstrated that *S. aureus* ATCC 29213 growth and enterotoxigenic

ability were hindered in co-culture with *E. faecalis* 41FL1 in skimmed milk at 30 °C for 24 h (Table 1). Our RNA-Seq data revealed considerable changes in the transcriptomic response of *S. aureus* in co-culture compared to single culture in the tested conditions. To our knowledge, this is the first study to present a comprehensive analysis of the alterations in the *S. aureus* transcriptome during growth in the presence of *E. faecalis* in skimmed milk.

Considering *S. aureus* genes upregulated or whose transcripts were found exclusively in co-culture at 7 h (Figure 2A; Table S1; Table S3), we identified an enrichment of transcripts coding for repair and detoxifying enzymes, chaperones, and several stress-induced transcriptional regulators. Of particular interest, we identified an upregulation of the following *S. aureus* genes in co-culture: *arcA* (expressed exclusively in co-culture at 7 h and upregulated by 2.63-fold at 24 h), *sigB* (7.99-fold), and *rex* (19.98-fold). The alternative sigma factor σ^B is involved in the response to a variety of environmental stresses in *S. aureus*, including pH-sensing transduction pathway (Gertz et al., 2000; Weinrick et al., 2004). The arginine deiminase ArcA, as part of the arginine deiminase (ADI) operon (*arcABDC*), catalyzes the hydrolysis of arginine into citrulline and ammonia, thus contributing to pH homeostasis and survival under non-lethal acid stress (Makhlin et al., 2007). Remarkably, *arcA* was shown to be upregulated in co-culture also at 24 h, as well as *arcD*, *arcB* and *arcC1*, a homolog for carbamate kinase found in the *S. aureus* ATCC 29213 genome outside of the ADI operon. Additionally, the redox sensing transcriptional factor Rex is known to be involved in the regulation of metabolic pathways that mediate NAD^+ regeneration and ATP synthesis in *S. aureus*, such as the ADI pathway, to assure survival and pathogenicity (Pagels et al., 2010). It has been previously described the strong ability of *E. faecalis* to reduce the redox potential and eliminate dissolved O_2 during growth in milk (Brasca et al., 2007; Morandi et al., 2016), a characteristic likely to have contributed to the generation of an oxygen-limiting environment in our co-culture conditions with profound implications over *rex* expression in *S. aureus*. Collectively, the upregulation of such genes by *S. aureus* in co-culture can be interpreted as part of a structured response to counteract the effects of pH- and

redox-induced stress caused by the presence of *E. faecalis*, also corroborated by the chemical changes in milk samples where *E. faecalis* was inoculated (Table 2).

At 24 h, COG enrichment analysis revealed an accumulation of transcripts belonging to the category of carbohydrate transport and metabolism in co-culture (Figure 2B). In particular, transcripts encoding enzymes involved in glycolysis and acetoin production (*alsSD* and *butA*) were significantly enriched in co-culture at 24 h (Figure 4), suggesting a shift towards fermentative metabolism in *S. aureus* in co-culture with *E. faecalis*. Interestingly, an upregulation of *alsSD* and *butA* in *S. aureus* grown in the presence of another LAB species (*L. lactis*) in a cheese matrix has been reported (Cretenet et al., 2011). In *S. aureus*, increased glycolytic activity and the activation of the acetoin biosynthetic pathway in environments rich in organic acids were shown to be a strategy to enhance survival by limiting metabolic-mediated cell death and lysis (Yang et al., 2006). In such cases, the generation of acetoin by *S. aureus* could be useful for NAD^+ regeneration, energy conservation and maintenance of intracellular pH homeostasis (Thomas et al., 2014). In support of these major alterations in the transcription of genes related to central carbon metabolism in co-culture, we also identified that transcripts of metabolite-responsive regulators known to be directly or indirectly involved in the regulation of the expression of several virulence factors in *S. aureus*, namely *codY* (Pohl et al., 2009; Roux et al., 2014), *rpiRA* and *rpiRB* (Zhu et al., 2011), accumulated in significantly larger amounts in co-culture at 24 h, as well as *ccpA* and *ccpE* at both 7 h and 24 h (Sonenshein, 2007, 2005) (Table 3; Table S1). In particular, CodY may act as a roadblock to transcription of *agr* P2 and P3 promoters (Majerczyk et al., 2010; Roux et al., 2014). Pathogenic bacteria use metabolite-responsive regulators to link metabolic status, energy homeostasis and synthesis of virulence determinants to the availability of biosynthetic intermediates derived from the glycolytic, pentose phosphate and TCA pathways (Richardson et al., 2015). It has been shown that *S. aureus* compromises its growth and pathogenic potential in favor of survival in conditions or environments where energy generation is limited (Somerville et al., 2002; Zhu et al., 2009) or

393 in competition with other microorganisms (Filkins et al., 2015; Orazi and O'Toole, 2017;
394 Ramsey et al., 2016) mainly by shifting towards fermentative growth and shutting down the
395 activity of energy consuming processes, such as virulence and *agr*-based quorum-sensing.
396 This previous knowledge help us explain, at least partly, our findings related to *S. aureus*
397 decreased growth, failed *agr* activation and overall altered transcriptomic profile in co-culture
398 with *E. faecalis*.

399 The pathogenicity of *S. aureus* involves the production of a wide array of cell wall-
400 attached and secreted proteins, whose expression is regulated at multiple levels in response to
401 cell density, energy availability, environmental and intracellular signals (Cheung et al., 2004;
402 Novick and Geisinger, 2008). In this context, *trans*-acting factors, such as sigma factors, two-
403 component systems (TCS) and metabolite-responsive elements, act as a dynamic network that
404 allows *S. aureus* to fine-tune its metabolism and virulence (Ibarra et al., 2013). The most
405 well-described and widespread TCS in *S. aureus* is the *agr* locus, which comprises four genes
406 (*agrBDCA*) and also functions as a quorum-sensing system whose intracellular effector is the
407 bifunctional RNAIII, a small regulatory RNA transcribed from the P3 promoter of the *agr*
408 system at higher cell densities (Novick et al., 1993; Novick and Geisinger, 2008). As an
409 opposing effector of the *agr* locus in the double-selector switch that governs *S. aureus*
410 virulence is found another transcriptional regulator, i.e. the Repressor of toxins (Rot)
411 (Bronesky et al., 2016). Rot simultaneously activates the transcription of genes encoding
412 several surface proteins and immunomodulators while repressing the transcription of
413 exotoxins, pore-forming toxins and exoenzymes (Saïd-Salim et al., 2003). *rot* activity occurs
414 at low cell density and its translation is repressed by RNAIII (Geisinger et al., 2006; Hsieh et
415 al., 2008). Thus, Rot and RNAIII have opposing roles in enabling *S. aureus* to phenotypic
416 switch between defense (adhesion, immune evasion and biofilm formation) and offensive
417 modes (degradation of host tissues and subversion of host defenses) (Nitzan et al., 2015). In
418 our study, we observed that the classical post-exponential activation of *agr* and its regulon
419 did not occur in *S. aureus* during growth in co-culture with *E. faecalis*, an observation

seemingly not related to cell density, since *S. aureus* population in co-culture easily reached the quorum sensing threshold (≥ 10^6 CFU/mL) (Table 1). Moreover, the transcription of *rot* was found to be upregulated in co-culture at 7 h by 2.46-fold while no *rot* transcripts were detected at 24 h (Table 3). Among Rot-regulated virulence genes (Saïd-Salim et al., 2003), we found an upregulation of *coa* and *sdrC* as well as a lack of transcripts coding for the serine protease operons (*splABCDEF* and *sspBC*) and urease complex (*ureABCDEF*) in co-culture transcriptome at 7 h (Table 3), which is consistent with the finding of higher amounts of *rot* transcripts under this condition. However, the number of transcripts coding for *hla* and *geh,* genes whose transcription is supposedly negatively regulated by Rot (Saïd-Salim et al., 2003), was found to be increased by 7.99-fold and 5.33-fold, respectively, in co-culture at 7 h, probably by *rot*- and/or *agr*-independent control mechanisms.

Enterotoxin production is one of the most important virulence-related traits of *S. aureus* for food safety concerns, since SEs are often implicated in foodborne intoxication outbreaks. We identified by means of whole genome sequencing the presence of genes coding for staphylococcal enterotoxin A (*sea*), enterotoxin P (*selp*) and the enterotoxin gene cluster (*seg*, *sen*, ψ*ent*$_1$, ψ*ent*$_2$, *sei*, *sem*, *seo*) in the genome of *S. aureus* ATCC 29213. Since the assessment of SE production in our single- and co-culture experiments included only the five classical enterotoxins, we thus focused on discussing the aspects concerning exclusively *sea* regulation in the genetic background of *S. aureus* ATCC 29213. *sea* is located on the genome of *Siphoviridae* temperate bacteriophages whose life cycle is characterized by two phases: the lysogenic and the lytic phase (Deghorain and Van Melderen, 2012). The expression of *sea* is not under the control of the *agr* system but related to the life cycle of the prophage; transition to the phage's lytic phase potentially activates and/or even enhances *sea* transcription (Sumby and Waldor, 2003; Tremaine et al., 1993). It has been shown that prophage induction leads to an increase in the amount of phage replicative form, as well as *sea* gene copies and transcripts, ultimately leading to an enhanced SEA production (Cao et al., 2012; Zeaki et al., 2015). More recently, it has been demonstrated that the alternative

447 sigma factor σ^H (*sigH*) promotes and stabilizes the lysogenization of several prophages in *S.*
448 *aureus* by upregulating the mRNA levels of prophage integrases (Tao et al., 2010).
449 Paralleling these observations with our findings, it is worth citing that *sigH* transcripts
450 accumulated significantly (> 15-fold) in *S. aureus* co-culture at 7 h compared to single
451 culture (Table 3), which possibly contributed to the lack of SEA in co-culture throughout the
452 incubation period.
453
454 **5. Conclusions**
455 Many studies have attempted to explore the interaction between pathogenic bacteria
456 and intrinsic positive microbiota in foods. In the present work, we discussed the alterations in
457 the *S. aureus* transcriptome during growth with *E. faecalis* in skimmed milk and provided
458 novel contributions to the current understanding of the LAB-*S. aureus* interaction in food-
459 mimicking conditions. However, we failed to determine a single explanation for our
460 observations, which are likely to have arisen as a result of a combined effect acidic and redox
461 stress with utmost negative impact on the expression of *S. aureus* virulence. Further
462 assessment of our transcriptomic findings at a proteomic level would be expected to provide
463 additional insights into this bacterial interaction, since transcript levels do not necessarily
464 correlate with the levels of its respective protein. Additionally, it would be of great interest to
465 investigate whether *S. aureus* replicates the rearrangement of its gene expression in similar
466 ways as described herein in the presence of a more complex microbiota and/or different
467 surrounding matrix.
468
469 **Acknowledgements**
470 This research was funded by the "Conselho Nacional do Desenvolvimento Científico
471 e Tecnológico – CNPq", Brazil [#485337/2012-6, 2012]. Gabriela Nogueira Viçosa is the
472 recipient of a Ph.D. fellowship from the "Coordenação de Aperfeiçoamento de Pessoal de
473 Nível Superior - CAPES" Foundation, Brazil [1529-13/7, 2013]. We also thank Francesca

474 Turroni and Gabriele Andrea Lugli from GenProbio, Parma, Italy for their support in the
475 analysis of RNA-Seq data.

476

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699

700 Table 1. Mean counts and standard deviation (MC ± SD) of *S. aureus* ATCC 29213 and *E.*
701 *faecalis* 41FL1 populations (log_{10} CFU/mL) and staphylococcal enterotoxin production in
702 single and co-culture experiments in sterile skimmed milk at 30 °C.

Incubation time (hours)	*S. aureus*				*E. faecalis*	
	Single culture		Co-culture		Single culture	Co-culture
	MC ± SD	SE production	MC ± SD	SE production		
0	3.33 ± 0.17	nd	3.10 ± 0.55	nd	6.26 ± 0.12	6.30 ± 0.06
4	3.91 ± 0.62	nd	4.65 ± 0.30	nd	7.29 ± 0.24	7.29 ± 0.16
7	7.17 ± 1.28	nd	6.01 ± 0.28	nd	8.12 ± 0.11	8.09 ± 0.18
12	8.28 ± 0.53[a]	nd	7.15 ± 0.13[b]	nd	8.82 ± 0.15	9.02 ± 0.23
24	8.66 ± 0.32[a]	detected*	7.49 ± 0.16[b]	nd	9.17 ± 0.11	9.22 ± 0.18

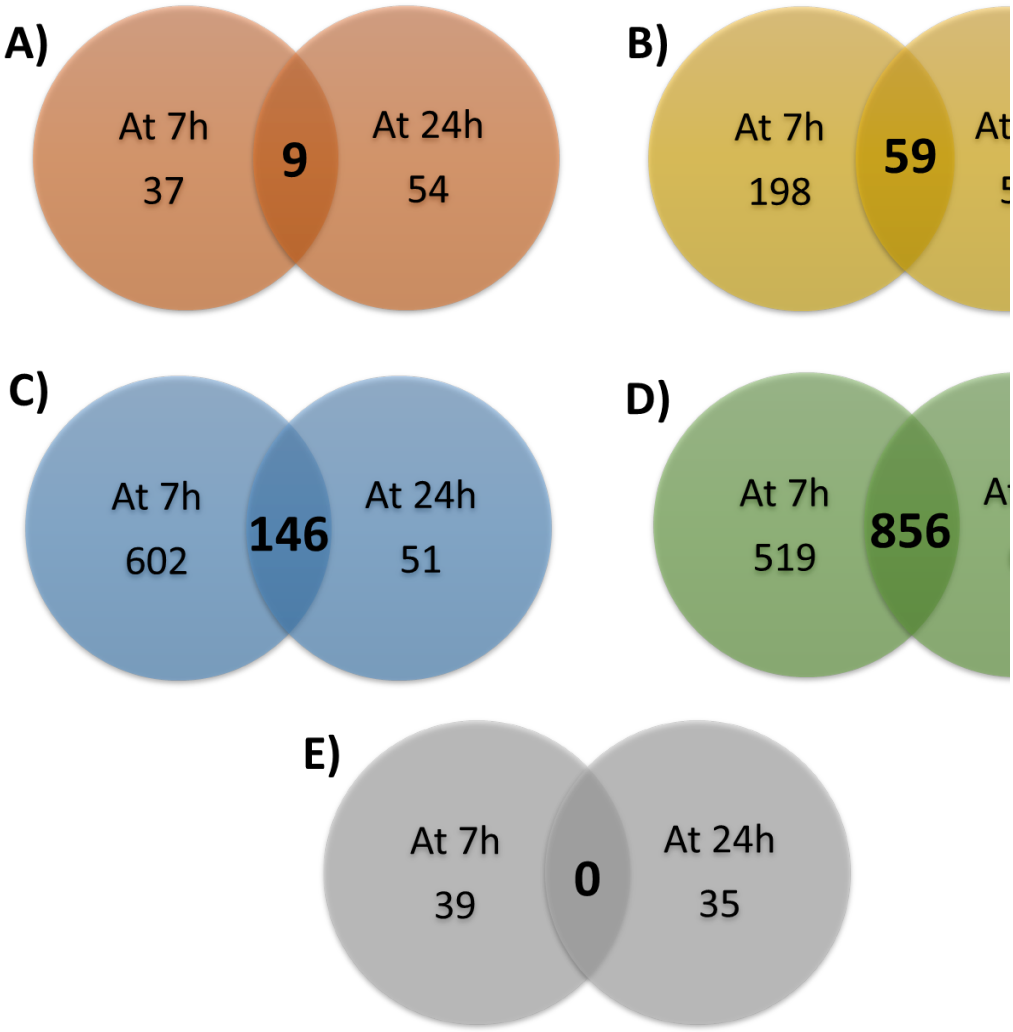
703 nd: not detected (below detection limit); *combined detection of all five classical
704 staphylococcal enterotoxins (SEA, SEB, SEC, SED, SEE); [a,b]Different letters in each line
705 indicate difference at 95% level of significance.

Table 2. pH, organic acids and sugar contents in skimmed milk during growth of *S. aureus* ATCC 29213 and *E. faecalis* 41FL1 alone or in combination at 30 °C for 24 h.

Chemical parameter	Treatment	Hours				
		0	**4**	**7**	**12**	**24**
pH	*E. faecalis*	6.53 ± 0.00[a]	6.36 ± 0.02[a]	6.19 ± 0.01[a]	5.77 ± 0.02[a]	4.94 ± 0.02[a]
	Co-culture	6.54 ± 0.00[a]	6.37 ± 0.00[a]	6.21 ± 0.09[a]	5.78 ± 0.02[a]	4.93 ± 0.04[a]
	S. aureus	6.57 ± 0.05[b]	6.79 ± 0.08[b]	6.72 ± 0.00[b]	6.73 ± 0.04[b]	6.81 ± 0.02[b]
	Significance	*	*	*	*	*
Lactose	*E. faecalis*	42.16 ± 0.19	42.01 ± 0.22	41.18 ± 0.38[a]	40.46 ± 0.38[a]	35.16 ± 0.20[a]
	Co-culture	41.77 ± 0.59	41.60 ± 0.50	40.69 ± 0.64[a]	39.64 ± 0.65[a]	35.13 ± 0.50[a]
	S. aureus	42.05 ± 0.21	41.91 ± 0.06	41.53 ± 0.17[b]	41.12 ± 0.09[b]	41.01 ± 0.37[b]
	Significance	ns	ns	*	*	*
Citric acid	*E. faecalis*	0.37 ± 0.01	0.36 ± 0.01	0.30 ± 0.01[b]	0.18 ± 0.01[b]	0.11 ± 0.06[b]
	Co-culture	0.37 ± 0.01	0.38 ± 0.01	0.26 ± 0.01[a]	0.14 ± 0.01[a]	0.09 ± 0.01[a]
	S. aureus	0.38 ± 0.01	0.37 ± 0.01	0.35 ± 0.01[c]	0.34 ± 0.01[c]	0.33 ± 0.01[c]
	Significance	ns	ns	*	*	*

Chemical parameter	Treatment	Hours				
		0	**4**	**7**	**12**	**24**
Pyruvic acid	*E. faecalis*	nd	nd	0.01 ± 0.00	0.02 ± 0.00[b]	0.03 ± 0.00[b]
	Co-culture	nd	nd	0.01 ± 0.00	0.02 ± 0.00[b]	0.03 ± 0.00[b]
	S. aureus	nd	nd	0.01 ± 0.00	0.01 ± 0.00[a]	0.01 ± 0.00[a]
	Significance	-	-	ns	*	*
Lactic acid	*E. faecalis*	0.04 ± 0.02[b]	0.14 ± 0.01[b]	0.33 ± 0.03[b]	1.73 ± 0.09[b]	5.45 ± 0.05[b]
	Co-culture	0.04 ± 0.01[b]	0.15 ± 0.01[b]	0.39 ± 0.01[c]	1.88 ± 0.02[c]	5.52 ± 0.02[b]
	S. aureus	nd[a]	nd[a]	nd[a]	nd[a]	0.08 ± 0.01[a]
	Significance	*	*	*	*	*
Acetic acid	*E. faecalis*	nd	0.02 ± 0.01[b]	0.13 ± 0.01[b]	0.28 ± 0.01[b]	0.40 ± 0.01[b]
	Co-culture	nd	0.05 ± 0.01[c]	0.13 ± 0.01[b]	0.30 ± 0.01[b]	0.40 ± 0.01[b]
	S. aureus	nd	nd[a]	nd[a]	0.02 ± 0.01[a]	0.03 ± 0.01[a]
	Significance	ns	*	*	*	*

[a,b,c] Different letters in the same column indicate difference at 95% level of significance; ns: not significant; nd: not detected (below detection limit).



710
711 Figure 1. Venn diagrams depicting the number of genes and their respective tr
712 response at 7 and 24 h of incubation at 30 °C in skimmed milk. A) Downregula
713 co-culture (LFC ≤ -1.0); B) Upregulated genes in co-culture (LFC ≥ +1.0); C)
714 transcripts not detected in single and co-culture (below detection limit); D)
715 transcripts absent only in co-culture (below detection limit); E) Genes wit
716 detected only in co-culture. LFC: $\log_2$ fold change.

717

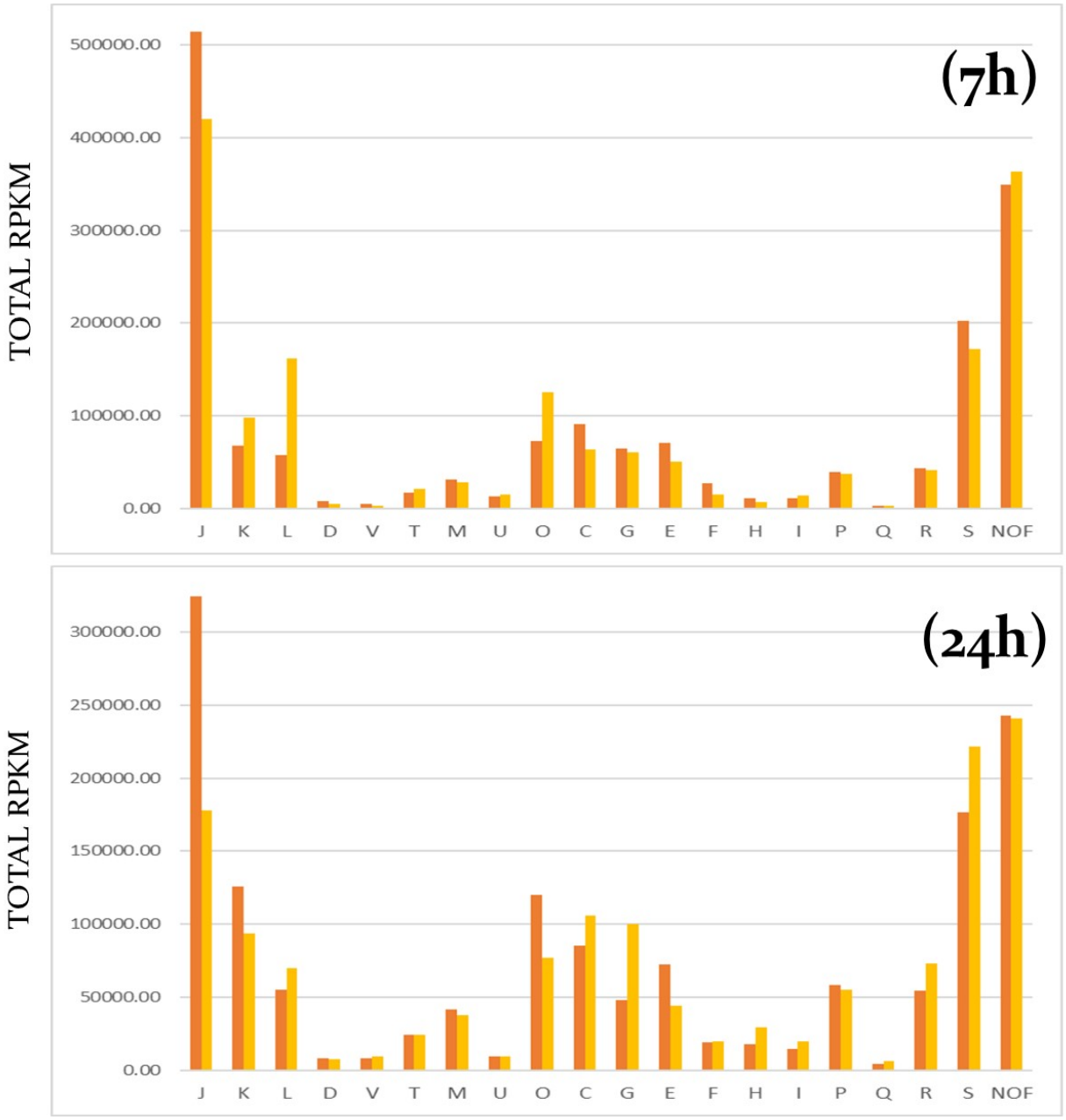
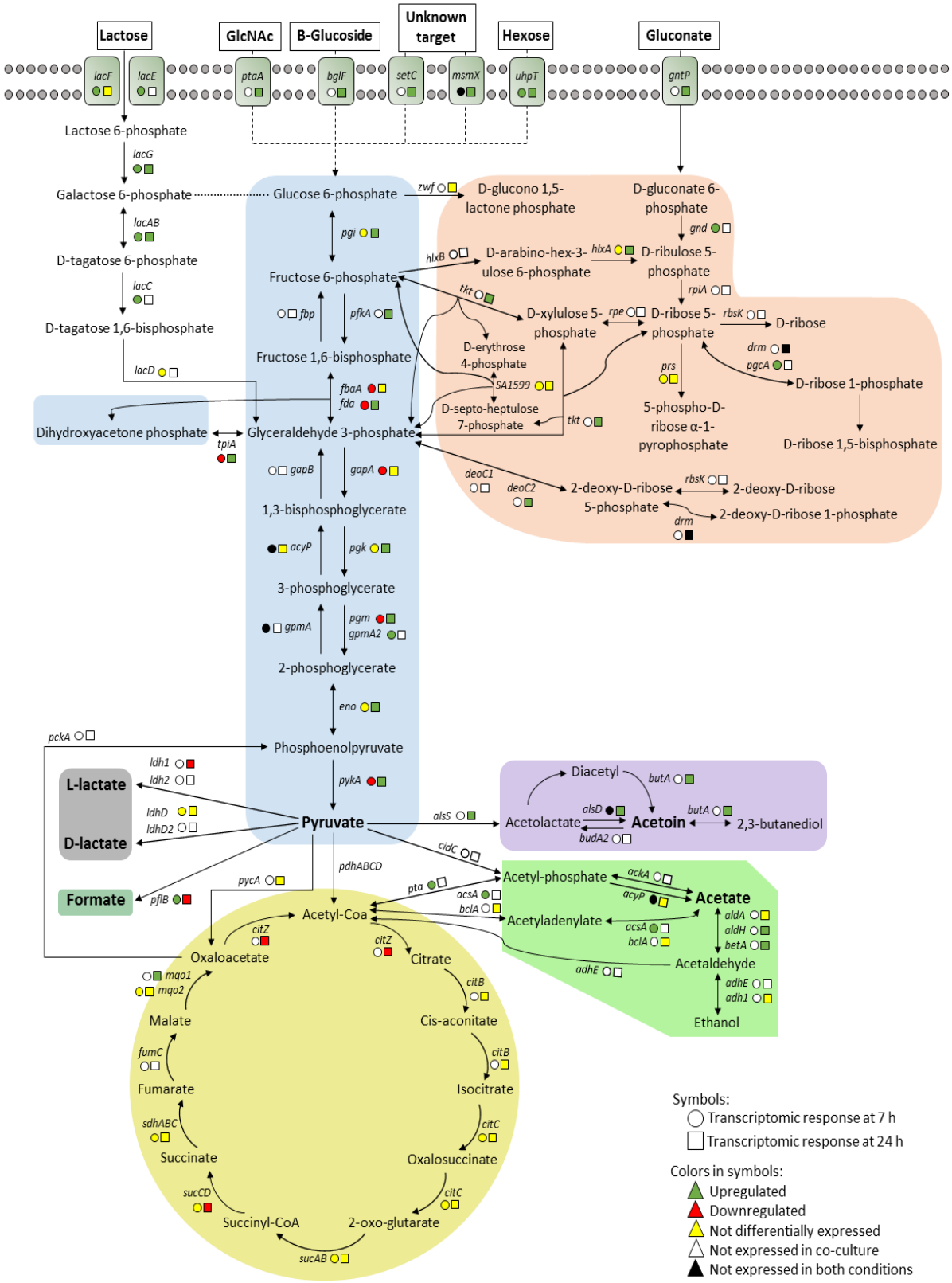


Figure 2. Overview of total abundance of *S. aureus* transcripts assigned to COG functional categories in single (orange) and co-culture (yellow) in skimmed milk at 7h and 24h of incubation. RPKM: reads per kilobase of transcript per million reads mapped. J: translation, ribosomal structure and biogenesis; K: transcription; L: replication, recombination and repair; D: cell cycle control, cell division, chromosome partitioning; V: defense mechanisms; T: signal transduction mechanisms; M: cell wall, membrane, envelope biogenesis; U: intracellular trafficking, secretion and vesicular transport; O: posttranslational modification, protein turnover, chaperones; C: energy production and conversion; G: carbohydrate transport and metabolism; E: amino acid transport and metabolism; F: nucleotide transport

and metabolism; H: coenzyme transport and metabolism; I: lipid transport and metabolism; P: inorganic ion transport and metabolism; Q: secondary metabolites biosynthesis, transport and catabolism; R: general function prediction only; S: function unknown; NOF: no orthologous found.



732 Figure 3. Transcriptional response of *S. aureus* genes related to central metabolic pathways
733 during growth with *E. faecalis* at 7 and 24 h of incubation. Genes involved in glucose and
734 lactose transport, glycolysis, gluconeogenesis, TCA cycle, pentose phosphate pathway,
735 acetate, lactate, ethanol and acetoin metabolism.

Table 3. Transcriptional response profile of virulence genes and transcriptional regulators of major relevance in *S. aureus* during growth in co-culture with *E. faecalis* at 7 and 24 h of incubation.

Function	**ORF denomination**	**Gene product**	**Gene**	**RPKM values at 7 h**		**LFC**	**RPKM values at 24 h**		**LFC**
				SC	**CC**		**SC**	**CC**	
Adherence and attachment to host tissues	ATCC29213_13_64	clumping factor A	*clfA*	1215.8	1733.0	0.5	1720.3	2469.0	0.5
	ATCC29213_46_136	clumping factor B	*clfB*	483.6	281.1	-0.8	238.3	934.4	2.0
	ATCC29213_46_5	fibronectin-binding protein FnbB	*fnbB*	31.9	254.8	3.0	406.6	529.4	0.4
	ATCC29213_46_6	fibronectin-binding protein FnbA	*fnbA*	88.5	0.0	NDCC	305.9	0.0	NDCC
	ATCC29213_21_1	extracellular matrix-binding protein Ebh	*ebh*	17.3	0.0	NDCC	58.3	134.2	1.2
	ATCC29213_21_45	elastin-binding protein EbpS	*ebpS*	503.9	1006.9	1.0	502.2	2092.0	2.1
	ATCC29213_14_90	extracellular adherence protein Eap	*eap*	105.9	0.0	NDCC	450.5	1407.5	1.6
	ATCC29213_10_37	serine-aspartate repeat-containing	*sdrC*	115.7	264.1	1.2	131.7	219.5	0.7

		protein C							
	ATCC29213_10_38	serine-aspartate rich fibrinogen/bone sialoprotein-binding protein D	*sdrD*	141.2	451.4	1.7	168.8	375.1	1.2
	ATCC29213_47_1	serine-aspartate repeat-containing protein E	*sdrE*	15.3	0.0	NDCC	28.5	305.8	3.4
	ATCC29213_11_1	serine-aspartate repeat-containing protein E	*sdrE*	31.3	0.0	NDCC	66.6	416.1	2.6
	ATCC29213_46_152	putative cell-wall-anchored protein SasF (LPXAG motif)	*sasF*	506.4	385.4	-0.4	243.5	961.0	2.0
	ATCC29213_45_10	surface protein G	*sasG*	0.0	0.0	NDBC	165.1	589.6	1.8
	ATCC29213_46_1	surface protein G	*sasG*	24.5	0.0	NDCC	195.6	489.0	1.3
	ATCC29213_51_1	surface protein G	*sasG*	0.0	0.0	NDBC	124.4	194.4	0.6
	ATCC29213_63_1	surface protein G	*sasG*	51.0	407.9	3.0	142.4	508.5	1.8
Immune evasion and	ATCC29213_17_21	alpha-hemolysin	*hla*	95.9	766.4	3.0	2242.5	0.0	NDCC
host damaging	ATCC29213_44_61	phospholipase C beta-hemolysin	*hlb*	0.0	0.0	NDBC	0.0	0.0	NDBC

enzymes	ATCC29213_42_85	gamma-hemolysin component A	*hlgA*	0.0	0.0	NDBC	164.6	0.0	NDCC
	ATCC29213_42_86	gamma-hemolysin component C	*hlgC*	0.0	0.0	NDBC	283.9	0.0	NDCC
	ATCC29213_42_87	gamma-hemolysin component B	*hlgB*	94.1	0.0	NDCC	125.1	0.0	NDCC
	ATCC29213_44_72	RNAIII (delta-hemolysin)	*hld*	0.0	0.0	NDBC	3738.7	0.0	NDCC
	ATCC29213_4_76	staphylocoagulase	*coa*	23.3	372.0	4.0	142.2	0.0	NDCC
	ATCC29213_13_68	thermonuclease	*nuc*	0.0	0.0	NDBC	124.7	0.0	NDCC
	ATCC29213_39_45	secretory antigen SsaA	*ssaA*	183.9	1470.0	3.0	464.3	0.0	NDCC
	ATCC29213_46_71	staphylococcal secretory antigen SsaA	*ssaA*	179.9	0.0	NDCC	15.9	2389.3	7.2
	ATCC29213_2_3	immunoglobulin G binding protein A precursor	*spa*	5373.7	1631.0	-1.7	153.6	1807.3	3.6
	ATCC29213_42_84	immunoglobulin G binding protein Sbi precursor	*sbi*	35.1	0.0	NDCC	1361.8	466.3	-1.55
	ATCC29213_31_2	serine protease SplF	*splF*	32.0	0.0	NDCC	68.0	0.0	NDCC
	ATCC29213_31_3	serine protease SplC	*splC*	0.0	0.0	NDBC	0.0	0.0	NDBC
	ATCC29213_31_4	serine protease SplB	*splB*	0.0	0.0	NDBC	84.6	0.0	NDCC

		ATCC29213_31_5	serine protease SplA	*splA*	65.0	0.0	NDCC	51.8	0.0	NDCC
		ATCC29213_70_1	serine protease SplD	*splD*	0.0	0.0	NDCC	102.0	0.0	NDCC
		ATCC29213_70_2	serine protease SplF	*splF*	32.5	0.0	NDCC	51.8	0.0	NDCC
		ATCC29213_14_153	staphostatin B	*sspC*	0.0	0.0	NDBC	111.4	0.0	NDCC
		ATCC29213_14_154	staphopain B	*sspB*	0.0	0.0	NDBC	41.4	517.3	3.64
		ATCC29213_33_49	staphopain A	*sspP*	0.0	0.0	NDBC	21.0	523.9	4.64
		ATCC29213_33_50	staphostatin A	*sspA_2*	0.0	0.0	NDBC	0.0	0.0	NDBC
		ATCC29213_39_30	urease subunit gamma	*ureA*	0.0	0.0	NDBC	80.9	0.0	NDCC
		ATCC29213_39_31	urease subunit beta	*ureB*	0.0	0.0	NDBC	89.4	0.0	NDCC
		ATCC29213_39_32	urease subunit alpha	*ureC*	53.6	0.0	NDCC	106.9	356.2	1.74
		ATCC29213_39_33	urease accessory protein UreE	*ureE*	0.0	0.0	NDBC	0.0	0.0	NDBC
		ATCC29213_39_34	urease accessory protein UreF	*ureF*	33.4	0.0	NDCC	35.5	443.3	3.64
		ATCC29213_39_35	urease accessory protein UreG	*ureG*	112.3	1197.1	3.41	179.1	0.0	NDCC
		ATCC29213_39_36	urease accessory protein UreD	*ureD*	55.0	0.0	NDCC	43.8	0.0	NDCC
		ATCC29213_4_165	glycerol ester hydrolase	*geh*	66.5	354.2	2.41	512.3	0.0	NDCC
ESAT-6	secretion	ATCC29213_4_127	virulence factor EsxA	*esxA*	34761.	15051.7	-1.2	11009.7	2084.9	-2.40

system				4					
	ATCC29213_4_128	type VII secretion protein EsaA	*esaA*	75.9	0.0	NDCC	84.7	201.7	1.25
	ATCC29213_4_129	secretion protein EssA	*essA*	100.4	0.0	NDCC	266.8	0.0	NDCC
	ATCC29213_4_130	type VII secretion protein EsaB	*esaB*	0.0	0.0	NDBC	0.0	0.0	NDBC
	ATCC29213_4_131	type VII secretion protein EssB	*essB*	86.2	0.0	NDCC	82.4	0.0	NDCC
	ATCC29213_4_132	type VII secretion protein EssC	*essC*	207.2	165.6	-0.3	96.3	0.0	NDCC
	ATCC29213_4_133	protein EsaC	*esaC*	117.3	0.0	NDCC	280.5	0.0	NDCC
	ATCC29213_4_134	virulence factor EsxB	*esxB*	146.4	0.0	NDCC	77.8	0.0	NDCC
Superantigens	ATCC29213_17_24	staphylococcal exotoxin 1	*set1*	0.0	0.0	NDBC	0.0	0.0	NDBC
	ATCC29213_17_25	staphylococcal exotoxin 4	*set4*	31.7	0.0	NDCC	33.7	0.0	NDCC
	ATCC29213_17_26	staphylococcal exotoxin 3	*set3*	95.1	0.0	NDCC	33.7	0.0	NDCC
	ATCC29213_5_5	staphylococcal exotoxin 6	*set6*	0.0	0.0	NDBC	35.9	0.0	NDCC
	ATCC29213_5_6	staphylococcal exotoxin 7	*set7*	66.2	1057.6	4.0	70.3	0.0	NDCC
	ATCC29213_5_7	staphylococcal exotoxin 8	*set8*	0.0	0.0	NDBC	137.0	0.0	NDCC
	ATCC29213_6_2	staphylococcal exotoxin 9	*set9*	0.0	0.0	NDBC	139.2	347.9	1.3
	ATCC29213_6_3	staphylococcal exotoxin 10	*set10*	0.0	0.0	NDBC	86.8	0.0	NDCC

	ATCC29213_6_4	staphylococcal exotoxin 11	*set11*	0.0	0.0	NDBC	140.7	0.0	NDCC
	ATCC29213_6_5	staphylococcal exotoxin 12	*set12*	0.0	0.0	NDBC	35.0	0.0	NDCC
	ATCC29213_6_6	staphylococcal exotoxin 13	*set13*	0.0	0.0	NDBC	35.0	0.0	NDCC
	ATCC29213_6_7	staphylococcal exotoxin 14	*set14*	0.0	0.0	NDBC	107.3	0.0	NDCC
	ATCC29213_7_3	staphylococcal exotoxin 15	*set15*	0.0	0.0	NDBC	107.3	0.0	NDCC
	ATCC29213_31_17	enterotoxin type G	*seg*	177.8	0.0	NDCC	78.7	0.0	NDCC
	ATCC29213_31_18	enterotoxin type N	*sem*	0.0	0.0	NDBC	16.2	0.0	NDCC
	ATCC29213_31_19	pseudoenterotoxin 2, ent2	*ψ ent 2*	116.4	0.0	NDCC	30.9	0.0	NDCC
	ATCC29213_31_20	pseudoenterotoxin 1, ent1	*ψ ent 1*	229.3	0.0	NDCC	0.0	0.0	NDBC
	ATCC29213_31_21	enterotoxin type I	*sei*	126.3	0.0	NDCC	50.4	839.1	4.1
	ATCC29213_31_22	enterotoxin type M	*sem*	64.0	0.0	NDCC	34.0	0.0	NDCC
	ATCC29213_31_23	enterotoxin type O	*seo*	0.0	0.0	NDBC	32.0	0.0	NDCC
	ATCC29213_34_23	enterotoxin type A	*sea*	416.4	0.0	NDCC	1027.5	790.3	-0.4
Global regulators	ATCC29213_44_73	accessory gene regulator protein AgrB	*agrB*	186.3	0.0	NDCC	435.7	0.0	NDCC
	ATCC29213_44_74	accessory gene regulator	*agrD*	321.6	0.0	NDCC	1025.4	0.0	NDCC

	autoinducing peptide AgrD							
ATCC29213_44_75	accessory gene regulator sensor histidine kinase AgrC	*agrC*	53.8	572.9	3.4	514.3	0.0	NDCC
ATCC29213_44_76	accessory gene regulator DNA-binding response regulator AgrA	*agrA*	321.1	0.0	NDCC	955.7	0.0	NDCC
ATCC29213_12_25	histidine protein kinase SaeS	*saeS*	109.0	0.0	NDCC	2212.2	0.0	NDCC
ATCC29213_12_26	two-component response regulator SaeR	*saeR*	335.1	0.0	NDCC	2796.5	890.5	-1.7
ATCC29213_11_54	HTH-type transcriptional regulator SarA	*sarA*	2274.5	3930.6	0.8	4018.6	3266.7	-0.3
ATCC29213_39_37	HTH-type transcriptional regulator SarR	*sarR*	861.3	4236.4	2.3	2394.5	0.0	NDCC
ATCC29213_2_4	HTH-type transcriptional regulator SarS	*sarS*	305.7	0.0	NDCC	130.0	0.0	NDCC
ATCC29213_42_51	HTH-type transcriptional regulator SarZ	*sarZ*	876.3	0.0	NDCC	1150.7	0.0	NDCC

	ATCC29213_46_2	HTH-type transcriptional regulator SarT	*sarT*	0.0	0.0	NDCC	68.6	0.0	NDCC
	ATCC29213_46_3	HTH-type transcriptional regulator SarU	*sarU*	0.0	0.0	NDCC	65.8	0.0	NDCC
	ATCC29213_39_10	HTH-type transcriptional regulator SarV	*sarV*	394.1	0.0	NDCC	768.1	0.0	NDCC
	ATCC29213_39_39	HTH-type transcriptional regulator SarY	*sarY*	61.9	0.0	NDCC	164.5	0.0	NDCC
	ATCC29213_11_103	HTH-type transcriptional regulator SarX	*sarX*	0.0	0.0	NDCC	0.0	0.0	NDBC
	ATCC29213_12_6	HTH-type transcriptional regulator MgrA	*mgrA*	1608.9	0.0	NDCC	1737.7	0.0	NDCC
	ATCC29213_28_117	HTH-type transcriptional regulator repressor of toxin Rot	*rot*	1490.7	3666.0	1.30	1645.5	0.0	NDCC
Metabolite-responsive regulators	ATCC29213_18_43	GTP-sensing transcriptional pleiotropic repressor CodY	*codY*	237.9	0.0	NDCC	284.5	790.3	1.47

	ATCC29213_28_91	catabolite control protein A	*ccpA*	162.7	743.2	2.1913	667.2	1853.0	1.4737
	ATCC29213_11_107	carbon catabolite responsive regulator CcpE	*ccpE*	159.3	848.8	2.4137	296.3	705.4	1.2513
	ATCC29213_4_162	RpiR family transcriptional regulator	*rpiRA*	57.5	0.0	NDCC	30.5	381.8	3.6
	ATCC29213_4_38	RpiR family transcriptional regulator	*rpiRB*	26.2	0.0	NDCC	153.1	695.8	2.2
	ATCC29213_40_7	RpiR family transcriptional regulator	*rpiRC*	210.9	0.0	NDCC	686.6	0.0	NDCC
Sigma factors	ATCC29213_24_10	RNA polymerase sigma factor A	*sigA*	124.7	996.9	2.9986	430.9	1933.1	2.1656
	ATCC29213_36_4	RNA polymerase sigma factor B	*sigB*	238.9	1909.2	2.9986	587.2	793.3	0.4342
	ATCC29213_36_5	serine-protein kinase RsbW	*rsbW*	96.0	1534.5	3.9986	153.1	0.0	NDCC
	ATCC29213_36_6	Anti-sigma factor B antagonist RsbV	*rsbV*	70.5	0.0	NDCC	562.2	0.0	NDCC
	ATCC29213_36_7	Sigma factor B regulation protein RsbU	*rsbU*	91.9	0.0	NDCC	134.3	0.0	NDCC

ATCC29213_10_10	RNA polymerase sigma factor H	*sigH*	80.8	1291.8	15.984	193.3	0.0	NDCC
ATCC29213_28_129	RNA polymerase sigma factor S	*rpoS*	48.9	0.0	NDCC	0.0	1299.7	NDPC

RPKM: reads per kilobase of transcript per million reads mapped

LFC: $\log_2$ fold change

SC: single culture

CC: co-culture

NDBC: not detected in both conditions (below detection limit)

NDCC: not detected in co-culture (below detection limit)

NDPC: not detected in pure culture (below detection limit)