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# Impact of *Staphylococcus aureus* infection on the late lactation goat milk proteome: new perspectives for monitoring and understanding mastitis in dairy goats

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Short title: **Goat milk proteins and *S. aureus***

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**Abstract**

The milk somatic cell count (SCC) is a standard parameter for monitoring intramammary infections (IMI) in dairy ruminants. In goats, however, the physiological increase in SCC occurring in late lactation heavily compromises its reliability. To identify and understand milk protein changes specifically related to IMI, we carried out a shotgun proteomics study comparing high SCC late lactation milk from goats with subclinical *Staphylococcus aureus* IMI and from healthy goats to low SCC mid-lactation milk from healthy goats. As a result, we detected 52 and 19 differential proteins (DPs) in *S. aureus*-infected and uninfected late lactation milk, respectively. Unexpectedly, one of the proteins higher in uninfected milk was serum amyloid A. On the other hand, 38 DPs were increased only in *S. aureus*-infected milk and included haptoglobin and numerous cytoskeletal proteins. Based on STRING analysis, the DPs unique to *S. aureus* infected milk were mainly involved in defense response, cytoskeleton organization, cell-to-cell, and cell-to-matrix interactions. Being tightly and specifically related to infectious/inflammatory processes, these proteins may hold promise as more reliable markers of IMI than SCC in late lactation goats.

*Significance*

The biological relevance of our results lies in the increased understanding of the changes specifically related to bacterial infection of the goat udder in late lactation. The DPs present only in *S. aureus* infected milk may find application as markers for improving the specificity of subclinical mastitis monitoring and detection in dairy goats in late lactation, when other widespread tools such as the SCC lose diagnostic value.

Keywords:

Goat mastitis; late lactation milk; *Staphylococcus aureus*; somatic cell count; shotgun proteomics; haptoglobin.

## 1. Introduction

Intramammary infections (IMI) and mastitis cause milk production losses and reduce dairy goat product quality. Subclinical mastitis due to chronic IMI can be especially problematic, and reliable monitoring and detection tools are needed for maintaining good profitability of goat productions [1]. The somatic cell count (SCC), that is, the number of cells per mL of milk, is largely considered a reliable IMI indicator in dairy ruminants [2]. In goats, however, the SCC is subjected to physiological variations related to age, parity, stage of lactation, estrus, and other factors [3–5], undermining specificity and limiting the diagnostic value of this practical and cost-effective marker. Late lactation, in particular, is associated with SCC increase in cow, sheep and goat milk [6], but the magnitude of this increase in goats is so high that SCC may not enable to distinguish infected from uninfected udders in late lactation [3,7,8]. Consequently, the reliability of the most widespread field tool, the California Mastitis Test (CMT), is severely affected [9]. The availability of a protein marker appearing in the milk only upon infection would increase the specificity of subclinical mastitis detection and support the screening of late lactation goats for IMI, enabling more meaningful management decisions especially at the dry-off [10,11].

The widespread adoption of milk SCC as an indicator of IMI is based on the notion that the number of cells in milk increases due to the active influx of neutrophils recalled into the milk as a result of the inflammation elicited by a microbial insult. Being this accompanied by increased permeability of the blood-milk barrier, with consequent leakage of serum contents into the milk, other ways to detect subclinical mastitis are based on these “leaked” proteins and other molecules found in the milk as a result of active secretion, cellular lysis or tissue rearrangements [11]. Investigating the proteome changes specifically associated with subclinical IMI is a suitable way to identify marker proteins that may represent a reliable alternative when the SCC loses specificity.

Gram-positive bacteria, and staphylococci in particular, are the most prevalent intramammary pathogens in dairy goats [12–15]. Gram-positive bacteria cause mainly subclinical, chronic infections that persist along the dry period [6,8] justifying the need for more sensitive and specific screening tools for monitoring mammary

gland health in dairy goats. Therefore, we selected *Staphylococcus aureus* subclinical IMI as the model condition for this study.

In summary, we applied a shotgun proteomics pipeline to compare late lactation, high SCC, *S. aureus* infected and uninfected milk with mid-lactation, low SCC uninfected milk to understand the changes induced by infection and to identify differential proteins with potential as subclinical mastitis markers in late lactation.

## 2. Materials and methods

### *2.1. Animals and milk samples*

Half-udder goat milk was retrieved from a frozen sample bank collected along the course of two entire lactations in a herd of Alpine goats farmed in Lombardy, Italy. All goats were clinically healthy for the two lactation years and showed no signs of mastitis. The detailed description of the herd and of experimental procedures was reported in a previous work [3]. Briefly, bacteriological analysis was carried out bi-monthly according to the National Mastitis Council standards [16] as described previously [17]. Ten µl of milk was spread on blood agar plates and incubated aerobically at 37°C. After 24 h, plates were examined, and colonies were provisionally identified based on Gram stain, morphology, and haemolysis patterns. Gram-positive cocci were tested for catalase and coagulase production for identification as *Staphylococcus aureus*, and colonies were re-isolated on Baird-Parker medium for further confirmation. Somatic cell count (SCC) was measured with an automated somatic cell counter (Bentley Somacount 150, Bentley Instrument, USA) [3]. Nine samples from multiparous goats were selected for the current study as follows: i) three mid-lactation samples (40±10 Days in milk - DIM) with very low SCC (19,000±7000) from half-udders producing a sterile milk bacterial culture for two consecutive samplings (MLU, Mid-lactation, Low SCC, Uninfected); ii) three late lactation samples (> 250 DIM) with SCC > 2,000,000 cells/mL (2,932,000±439,000) from half-udders producing a sterile milk bacterial culture for the whole lactation (LHU, Late lactation, High SCC, Uninfected); and iii) three late lactation samples (> 250 DIM) with SCC > 2,000,000 cells/mL (3,980,000±74,000) from goat half-udders with a milk bacterial culture repeatedly positive for *S. aureus* in the previous lactation year (LHS, Late lactation,

High SCC, *Staphylococcus aureus* infected). *S. aureus* positive goats were culled at the end of lactation in the first year. The whole herd tested negative to *S. aureus* in the second year, when MLU and LHU samples were collected. The SCC > 2,000,000 cells/mL threshold was selected because the California Mastitis Test (CMT) scores are the highest over this value.

*2.2. Milk sample preparation for proteomic analysis*

Milk sample preparation for proteomic analysis was carried out as described previously [17]. Briefly, milk was allowed to thaw at room temperature and centrifuged at 800 x g at 4°C for 10 min, the fat ring was removed, and skim milk was diluted 1:1 with lysis buffer, incubated at 95°C for 10 min and sonicated in a refrigerated water bath for 10 min, after which the suspension was centrifuged at 10.000 x g for 10 min at 4°C. Then, 7 µl of extract was subjected to filter-aided sample preparation (FASP) [18]. Protein samples were reduced, alkylated, and digested with trypsin on 3 kDa cut-off Amicon Ultra-0.5 mL centrifugal filter units (Millipore, Billerica, MA, USA). Peptide concentration was determined with a NanoDrop 2000 spectrophotometer (Thermo Scientific, San Jose, CA, USA).

*2.3. Tandem mass spectrometry analysis of peptides*

All peptide mixtures were analysed on a Q-Exactive interfaced with an UltiMate 3000 RSLCnanoLC system (Thermo Scientific, San Jose, CA, USA), as detailed previously [19], using 4 µg of peptide mixture. Protein identification was carried out with Proteome Discoverer (version 1.4; Thermo Scientific) and Sequest-HT as the search engine. MS/MS spectra were analyzed as follows. Database: custom, obtained by merging *Bos taurus, Capra hircus* and *Staphylococcus* databases. These were downloaded from Swiss-Prot (*Bos taurus*) and TrEMBL (*Capra hircus* and *Staphylococcus)* release2017_05 and 2016_11, respectively; enzyme: trypsin, with two missed cleavages allowed; precursor mass tolerance: 10 ppm; MS/MS tolerance: 0.02 Da; charge states: +2, +3, and +4; cysteine carbamidomethylation as static modification and methionine oxidation as dynamic modifications. The percolator algorithm was used for protein significance and for peptide validation

(false discovery rate, FDR, < 0.01). Peptide and protein grouping according to the Proteome Discoverer's algorithm were allowed, applying the strict maximum parsimony principle.

*2.4. Proteomic data analysis*

Protein abundance changes were assessed by the spectral counting (SpC) approach as described previously (Pisanu et al., 2019). When proteins had more than one entry, only those with the highest number of unique peptides and SpCs were considered. Only proteins identified in at least two biological replicates and having ≥ 2 SpCs (Peptide Spectrum Matches, PSMs) in at least one sample of the group were considered for differential analysis. Relative abundance of single proteins in all samples and abundance changes of proteins between groups were calculated by considering the normalised spectral abundance factor (NSAF) and the $R_{SC}$ (the log2 of the protein abundance ratio), respectively [20,21]. Statistical significance was assessed by the beta-binomial test with FDR correction according to Benjamini-Hochberg [22]. Only proteins with $R_{SC} \geq 1.0$ or $\leq -1.0$ between the compared groups and having a p-value $\leq 0.05$ were considered differential. The biological processes and molecular functions reported by UniProtKB database were used for gene ontology (GO) analysis of differential proteins (DPs), integrated with manual curation. Protein-protein interaction network was assessed with the STRING database (Version 11, http://string-db.org/), after replacing all *Capra hircus* UniProt IDs with the corresponding *Bos taurus* UniProt IDs using the Basic Local Alignment Search Tool (BLAST) [23] and by taking into account only functional interactions with high confidence (combined score > 0.7) [24].

*2.5. Data Availability*

The data have been deposited to the ProteomeXchange with identifier PXD017243 [25]. A complete description of the dataset is available in Pisanu et al., 2020 (Data in Brief, submitted).

## 3. Results

### *3.1. Shotgun proteomics and differential analysis*

*S. aureus* infected and uninfected milk was subjected to a shotgun proteomics workflow combining FASP, RP-HPLC, and high-resolution orbitrap MS. This led to the identification of 540 total unique proteins, of which 256 eligible for differential analysis. The complete description of the proteomic datasets is available in Pisanu et al. 2020 (Data in Brief, submitted).

To identify the changes specifically induced by *S. aureus* as opposed to the physiological changes occurring in late lactation, we compared late lactation, high SCC infected and uninfected milk with mid-lactation, low SCC uninfected milk. As a result, late lactation infected milk showed 52 significant DPs, while late lactation uninfected milk showed only 19 DPs. Results are summarised in Table 1 and are detailed in Supplementary file (sheets 1 and 2, respectively). The higher number of DPs in *S. aureus* positive milk indicated that the presence of *S. aureus* was more impacting on the milk proteome than the physiological late lactation changes alone.

Table 2 lists all the DPs obtained in the two comparisons with the respective $\log_2$ ratio abundance values ($R_{SC}$). Protein abundance changes were generally more intense in *S. aureus* infected milk, as most common DPs showed higher $R_{SC}$ values in this sample group. The top DPs in both *S. aureus* infected and uninfected milk were lactotransferrin and cathelicidin-2. Vimentin, the third top DP, changed significantly only in *S. aureus* infected milk. In uninfected milk, only complement C3, olfactomedin-like protein 3, and serum amyloid A showed higher $R_{SC}$ values, and only three DPs were unique: fatty acid synthase, calreticulin, and lactoperoxidase. On the other hand, 38 proteins showed significant changes only in *S. aureus* infected milk and are highlighted in bold in Table 2.

### *3.2. Functional analysis*

The 38 DPs unique to *S. aureus* infected milk (Table 2, bold) were analyzed for their interactions and biological functions by STRING. Several proteins were strongly connected, such as tubulins with 14-3-3 proteins and

heat-shock proteins, myosin light chains, other cytoskeletal proteins, and proteins involved in cell-to-cell and cell-to-matrix interactions.

Supplementary file, sheets 4-7, report the list of significant GO terms and pathways enriched for the categories Biological Process, KEGG Pathways, Cellular Component, INTERPRO Protein Domains and Features, and Reactome Pathways, respectively. The most relevant Biological Process GO terms and KEGG pathways are indicated in Figure 1A and in Figure 1B.

## 4. Discussion

Aim of this work was to detect and understand milk changes specifically related to IMI in dairy goats, especially focusing on late lactation, by differential label-free shotgun proteomics. This was also the first proteomic investigation of milk from goats with subclinical *S. aureus* mastitis.

When comparing the DPs observed in late lactation *S. aureus* infected and uninfected milk, the number, identity, and abundance indicated that the presence of *S. aureus* had a specific and strong impact on the goat milk proteome. Although some DPs, especially those with the highest $R_{SC}$ values, were increased in both infected and uninfected milk, most were detected only in infected milk and are the most relevant for understanding the differences between the two conditions and for identifying useful mastitis markers.

Lactotransferrin, cathelicidins, serum amyloid A, and haptoglobin are long known to increase in cow milk during mastitis [10,26–32] and have been evaluated as protein markers also in sheep and goats [3,33–35]. In this study, lactotransferrin and cathelicidins increased in late lactation *S. aureus* infected milk but increased also in late lactation uninfected milk, although at a slightly lower extent. These proteins might increase in the milk as a result of neutrophil influx, and as opposed to other dairy species, in goats this occurs also physiologically as lactation progresses [5]. This is in line with the observations recently made by our group when comparing the value of cathelicidins in late lactation sheep and goats [3] and, in spite of the great value in other dairy species, might reduce mastitis detection specificity in late lactation goats.

On the other hand, the unexpected inverse behavior of serum amyloid A and haptoglobin was of significant interest. In fact, previous gel-based and gel-free proteomic studies carried out in goat milk are contrasting in

193 this respect. Olumee-Shabon et al. [36] observed a significant increase of both haptoglobin and serum
194 amyloid A in agreement with previous studies in cows, while Wang et al. detected serum amyloid A but not
195 haptoglobin [37]. However, both studies evaluated an experimentally induced lipopolysaccharide (LPS)
196 mastitis, and specificities in the host response to Gram-negative and Gram-positive microorganisms might
197 partly account for these differences [38,39]. This is especially relevant when considering that Gram-positive
198 bacteria are by far the leading intramammary pathogens in dairy goats, with *Staphylococcus* being the most
199 prevalent genus [12–15]. *Staphylococcus* spp. also cause mainly chronic, subclinical infections that persist
200 along the dry period [8] justifying the need for more sensitive and specific screening tools. In our study, serum
201 amyloid A was increased in high SCC milk, but such increase was not specific for the presence of an infection;
202 actually, the $R_{SC}$ value was higher in uninfected (2.41) than in *S. aureus* infected (1.21) milk, raising the
203 question that it might be related to physiological rather than pathological processes. On the other hand,
204 haptoglobin increased significantly only in *S. aureus* infected milk ($R_{SC}$ = 1.70). Therefore, haptoglobin might
205 have potential as a specific mastitis marker also in late lactation, high SCC goat milk. A dedicated study with
206 large sample numbers, different etiological agents, and thoroughly validated antibodies will be needed to
207 further investigate this finding.
208 The STRING protein network analysis provided interesting information on the biological processes and
209 pathways involving the 38 DPs detected only in *S. aureus* infected milk. Notably, tight junction, regulation of
210 the actin cytoskeleton and leukocyte transendothelial migration were among the most significant KEGG
211 pathways highlighted by STRING analysis. Tight junctions participate actively in regulating the passage of
212 blood-derived antimicrobial factors, cytokines, and neutrophils [40,41]. Accordingly, loss of tight junction
213 integrity has been linked to reduced milk secretion and increased paracellular mixture of serum and milk
214 components [42,43]. The specific function of haptoglobin in the context of mammary gland inflammation is
215 mainly attributed to hemoglobin scavenging to inhibit its oxidative activity [44], but the full-length precursor
216 of haptoglobin, zonulin, increases epithelial permeability by mediating intercellular tight junction
217 disassembly [45]. Altogether, this suggests that its increase only in late lactation infected milk might be a

specific consequence of the blood-milk barrier dynamics related to inflammation [42,43,46–48] and highlights its potential as specific goat mastitis marker.

The highest $R_{SC}$ value for proteins increased only in *S. aureus* infected milk was observed for vimentin, a highly abundant intermediate filament protein [49] involved in the innate immune response to pathogens [50] by regulating inflammasome activity [51]. Vimentin was one of the top 15 up-regulated proteins at 57, 81, and 312 hours after intramammary challenge of cows with *Streptococcus uberis* [52], and it was the first DP in the milk of buffaloes with *S. aureus* IMI [17]. Several members of the annexin family were also significantly higher in *S. aureus*-infected milk. Annexins are involved in vesicular trafficking and might be increased as a result of cell degranulation, especially by neutrophils [59]. Interestingly, clumping factor A of *S. aureus* interacts with annexin A2 on mammary epithelial cells mediating its entry into the host cell [60]. The increase in apolipoprotein A4 only in infected milk is in line with the observations of Olumee-Shabon *et al.* in the milk of goats challenged with LPS [36].

Other DPs increased also in late lactation, uninfected milk. Among other causes, these might be the result of physiological processes involved in mammary gland tissue dynamics and recycling associated with the natural involution of the mammary gland at the cessation of lactation [6,53].

In conclusion, this work provided the first characterization of *S. aureus* infected goat milk; identified the differences between infected and uninfected late lactation, high SCC milk; identified several proteins that are increased in milk only upon infection; provided insights on the mechanisms leading to the specific changes found in the milk proteome when an IMI is present; and, most importantly, identified putative markers that might improve specificity of subclinical mastitis detection and enable more meaningful management decisions especially in late lactation, when the diagnostic value of SCC is reduced.

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## Author contributions

Proteomic analysis and differential proteomics: SP, CC, DP. Microbiological analysis of milk: CP, MP. Animal examination, sample collection: VB. Funding and contribution to study design: SU, VB. Study conception, design and coordination, data analysis and interpretation, manuscript drafting: MFA. Data interpretation and manuscript revision: All authors.

## Conflict of interest

The authors declare no conflict of interest.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at

https://www.sciencedirect.com/science/article/pii/S1874391920301317?via%3Dihub.

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## Figure Legends

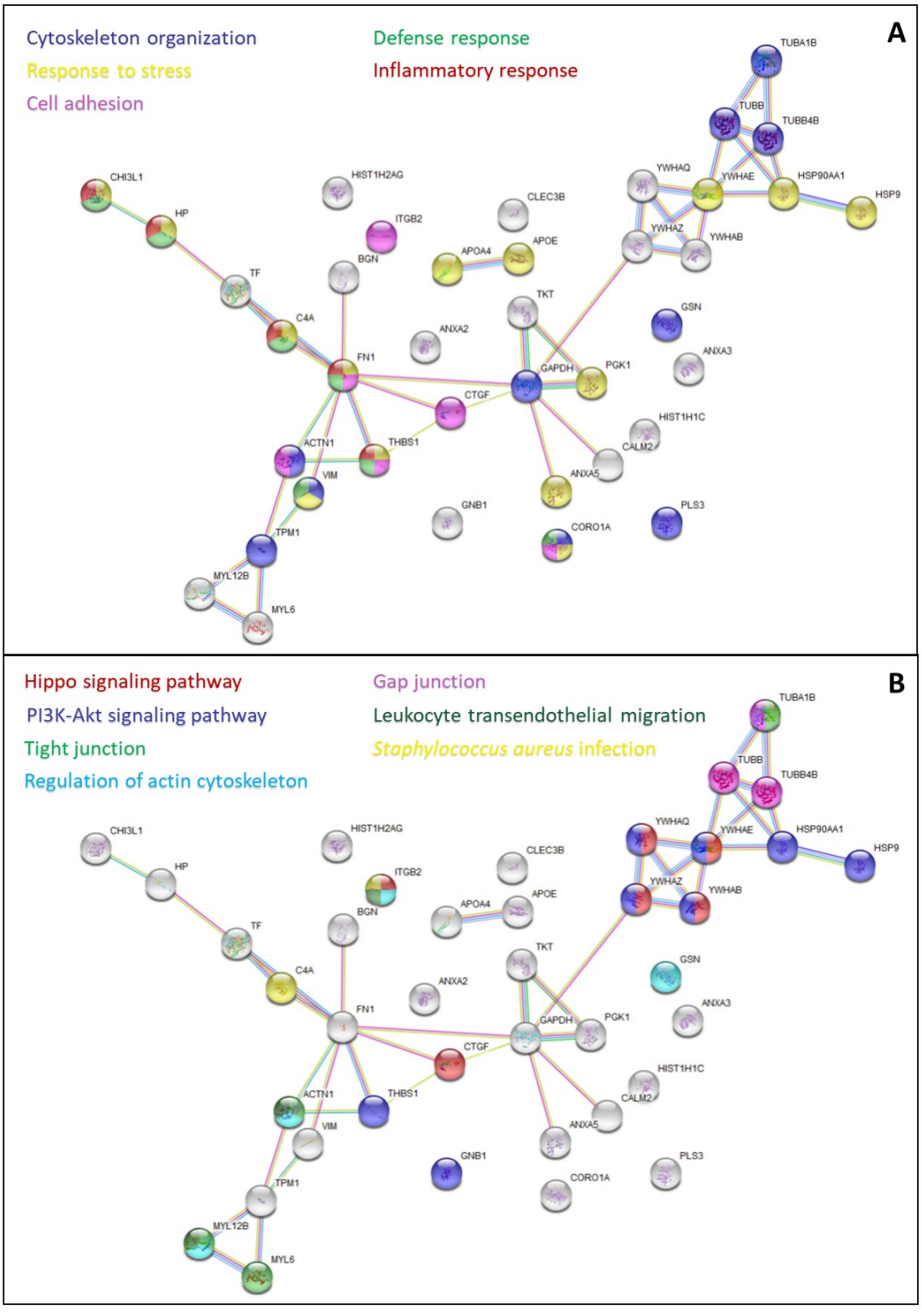



**Figure 1. STRING interaction networks of the proteins significantly changed only in *S. aureus* infected milk.** Proteins associated with relevant statistically significant Biological Process GO terms (**A**) and KEGG Pathways (**B**) are marked with different colors as indicated. Gene names correspond to the proteins reported in Table 2.

**TABLES**

**Table 1. Summary of differential proteomic results.**

| | Eligible for comparison* | Changed** | Differential*** $R_{SC} \leq -1.0$ or $\geq 1.0$ | Increased*** $R_{SC} \geq 1.0$ | Decreased*** $R_{SC} \leq -1.0$ |
|---|---|---|---|---|---|
| Infected[b] | 243 | 62 | 52 | 52 | 0 |
| Uninfected[b] | 152 | 20 | 19 | 18 | 1 |

[a]Late lactation *S. aureus* infected milk vs mid-lactation uninfected milk. [b]Late lactation uninfected milk vs mid-lactation uninfected milk. *Proteins identified in at least two biological replicates and with ≥2 spectral counts in at least one sample of the experimental group. **$p \leq 0.05$ by the beta-binomial test with FDR correction according to Benjamini-Hochberg. *** $p \leq 0.05$ by the beta-binomial test with FDR correction according to Benjamini-Hochberg and $R_{SC} \leq -1.0$ or $\geq 1.0$.

**Table 2. Significantly differential proteins observed in late lactation *S. aureus* infected and uninfected milk in comparison to mid-lactation uninfected milk.** The respective $R_{SC}$ values are reported for all the differential proteins in the two comparisons. Bold: proteins significantly increased only in infected milk.

| Accession | Gene name | Description | Infected[a]* | Uninfected[b]* |
|---|---|---|---|---|
| Q29477 | LTF | Lactotransferrin | 4.22 | 4.05 |
| P82018 | CATHL2 | Cathelicidin-2 | 3.30 | 2.75 |
| P48616 | VIM | Vimentin | **3.01** | - |
| Q9XSJ4 | ENO1 | Alpha-enolase | 2.98 | 1.90 |
| P46193 | ANXA1 | Annexin A1 | 2.84 | 2.24 |
| P62808 | HIST1H2B | Histone H2B type 1 | 2.74 | 2.12 |
| P60712 | ACTA1 | Actin, cytoplasmic 1 | 2.49 | 1.74 |
| Q8SPQ0 | CHI3L1 | Chitinase-3-like protein 1 | 2.38 | 2.12 |
| P62803 | H4 | Histone H4 | 2.37 | 1.71 |
| Q2UVX4 | C3 | Complement C3 | 2.35 | 2.62 |
| P07589 | FN1 | Fibronectin | **2.33** | - |
| Q28178 | THBS1 | Thrombospondin-1 | **2.33** | - |
| A5D7D1 | ACTN4 | Alpha-actinin-4 | 2.24 | 1.10 |
| P02584 | PFN1 | Profilin-1 | 2.15 | 1.10 |
| P68138 | ACTA1 | Actin, alpha skeletal muscle | 2.10 | 1.23 |
| Q2KJD0 | TUBB | Tubulin beta-5 chain | **2.10** | - |
| Q3MHM5 | TUBB4B | Tubulin beta-4B chain | **2.05** | - |
| P62871 | GNB1 | Guanine nucleotide-binding protein G(I)/G(S)/G(T) sub β-1 | **2.00** | - |
| Q3B7N2 | ACTN1 | Alpha-actinin-1 | **2.00** | - |
| Q5VI41 | ITGB2 | Integrin beta-2 | **2.00** | - |
| P10096 | GAPDH | Glyceraldehyde-3-phosphate dehydrogenase | **1.88** | - |
| A7E3Q8 | PLS3 | Plastin-3 | **1.88** | - |
| Q71SP7 | FASN | Fatty acid synthase | - | 1.85 |
| Q3SWX7 | ANXA3 | Annexin A3 | **1.82** | - |
| P31976 | EZR | Ezrin | 1.76 | 1.65 |
| Q2TBU0 | HP | Haptoglobin | **1.70** | - |
| P63103 | YWHAZ | 14-3-3 protein zeta/delta | **1.70** | - |
| P60661 | MYL6 | Myosin light polypeptide 6 | **1.63** | - |
| P68250 | YWHAB | 14-3-3 protein beta/alpha | **1.56** | - |
| Q5E956 | TPI1 | Triosephosphate isomerase | 1.70 | 1.56 |
| P02253 | HIST1H1C | Histone H1.2 | **1.48** | - |
| Q3SZI4 | YWHAQ | 14-3-3 protein theta | **1.48** | - |
| O18739 | CTGF | Connective tissue growth factor | **1.48** | - |
| P81947 | TUBA1B | Tubulin alpha-1B chain | **1.48** | - |
| Q0VCP3 | OLFML3 | Olfactomedin-like protein 3 | 1.23 | 1.45 |
| Q76LV2 | HSP90AA1 | Heat shock protein HSP 90-alpha | **1.42** | - |
| P81287 | ANXA5 | Annexin A5 | **1.40** | - |
| Q3SX14 | GSN | Gelsolin | **1.38** | - |
| Q32PJ2 | APOA4 | Apolipoprotein A-IV | **1.38** | - |
| Q3T0P6 | PGK1 | Phosphoglycerate kinase 1 | **1.32** | - |
| Q92176 | CORO1A | Coronin-1A | **1.32** | - |
| P21809 | BGN | Biglycan | **1.23** | - |
| Q76LV1 | HSP90AB1 | Heat shock protein HSP 90-beta | **1.23** | - |
| P62157 | CALM2 | Calmodulin | **1.23** | - |
| Q03247 | APOE | Apolipoprotein E | **1.22** | -. |
| P35541 | SAA2 | Serum amyloid A protein | 1.21 | 2.41 |
| P62261 | YWHAE | 14-3-3 protein epsilon | **1.14** | - |

| Accession | Gene name | Description | Infected[a]* | Uninfected[b]* |
|---|---|---|---|---|
| P04272 | ANXA2 | Annexin A2 | **1.14** | - |
| P0C0S9 | HIST1H2AG | Histone H2A type 1 | **1.14** | - |
| Q29443 | TF | Serotransferrin | **1.13** | - |
| P52193 | CALR | Calreticulin | - | 1.10 |
| A4IF97 | MYL12B | Myosin regulatory light chain 12B | **1.04** | - |
| Q6B855 | TKT | Transketolase | **1.04** | - |
| P01030 | C4 | Complement C4 | **1.04** | - |
| Q2KIS7 | CLEC3B | Tetranectin | **1.04** | - |
| Q5KR47 | TPM1 | Tropomyosin alpha-3 chain | **1.04** | - |
| P80025 | LPO | Lactoperoxidase | - | -1.04 |

[a]Late lactation, high SCC, *S. aureus*-infected milk vs mid-lactation, low SCC, uninfected milk. [b]Late lactation, high SCC, uninfected milk vs mid-lactation, low SCC, uninfected milk. *$R_{SC}$ ≥ 1.0 or ≤ -1.0 and p-value ≤ 0.05 with FDR correction according to Benjamini-Hochberg.