Article

The relationship between S. aureus and branched-chain amino acids content in composite cow milk

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Simple Summary: Staphylococcus aureus is not only a common cause of bovine mastitis, but also an agent of food poisoning in humans. To infect hosts S. aureus must either take up nutrients from the environment or produce them itself. S. aureus in fact has the ability to produce branched-chain amino acids (BCAAs) under certain nutritional conditions. We show that levels of two BCCA (leucine and isoleucine) are correlated to the load of S. aureus in composite milk samples.

Abstract: The early diagnosis of mastitis represents an essential factor for a prompt detection of the animal for further actions. In fact, if not culled, infected cows must be segregated from the milking herd and milked last, or milked with separate milking units. Besides microbiological analysis, the somatic cell count (SCC) commonly used as predictor of intramammary infection, frequently lead to a misclassification of milk samples. To overcome these limitations, more specific biomarkers are continuously evaluated. Total amino acid content increases significantly in mastitic milk compared to normal one. Bovine mastitis can arise as a result of infection of the mammary gland by Staphylococcus aureus. Multiplication of this bacterium within the mammary gland is required for infection to persist. S. aureus requires branched-chain amino acids (BCAAs: isoleucine, leucine, valine) for protein synthesis, branched-chain fatty acids synthesis and environmental adaptation by responding to their availability via transcriptional regulators. The importance of BCAAs for S. aureus physiology necessitates that it either synthesize them or scavenge them from the environment. Increase of BCAAs in composite milk has been postulated to be linked to mammary infection by S. aureus. In the present work, we demonstrated, by a direct ion-pairing reversed-phase method based on the use of the evaporative light-scattering detector (IP-RP-HPLC-ELSD), applied to 65 composite cow milk samples, a correlation between the concentration of isoleucine and leucine and S. aureus load.

Keywords: branched-chain amino acid; ion-pair reversed-phase liquid chromatography; mastitis; dairy cow; Staphylococcus aureus

1. Introduction

Staphylococcus aureus causes one of the most common types of chronic mastitis. Though some cows may be up with clinical mastitis (especially after calving), the infection is usually subclinical, often with no detectable changes in milk or the udder. The bacteria have the ability to survive and

multiply in the mammary gland tissues and are contagious. When the infection is established is hard to treat with antimicrobial therapy and the infected subjects must be divided from the rest of the herd in order to avoid the spread of the bacteria that usually occurs at milking time (Grispoldi et al., 2019). It is well known that using standard milking-time hygiene techniques in herds with low level of SCC (<200,000 cells/mL) is hard to eradicate the presence of S. aureus [1-3]. Schukken, et al. [4] have shown that 3% of all animals are infected with S. aureus. However, S. aureus represents 10 to 12% of all clinical mastitis infections [5].

According to the EU Regulation (Regulation (EC) No 853/2004 of the European parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin) raw milk must meets the following criteria: plate count at 30 °C ≤ 100,000 colony forming unit (cfu)/mL and somatic cell count ≤ 400,000 cells/mL. However, since several factors (i.e. animal age, breed, stage of lactation, the time of the day at sampling, the daily frequency of milking) can influence this value, some perplexities are correlated to the exclusive use of SCC to diagnose subclinical infection.

Interestingly, cows infected with S. aureus do not necessarily have elevated SCC. Jones at al. (1984) demonstrated that only 60% of cows infected with S. aureus produced milk with more than 300,000 somatic cells/mL. There are evidences that a percentage between 12 and 15% of heifers are infected with S. aureus after the first calving [6-8]. If not recognized and left untreated they produce less milk and remain infected, acting as reservoirs [9]. Although as many as half of the cows with high SCC may be infected with S. aureus, SCC-based test alone is not sensitive enough to positively diagnose S. aureus infections [3,10,11].

In this scenario, the early diagnosis of mastitis, especially at subclinical stages, becomes a pivotal factor for a prompt consideration and detection of the animal, thus minimizing the related consequences in term of transmission of infection. While it's easy to diagnose clinical forms by observing the symptoms and the alterations of some milk characteristics such as color, flavor and taste, it's much harder to diagnose subclinical forms, in particular during the early stages of the infection. Indeed, in the latter case, clinical signs are not evident (milk appears normal) and the use of specific tests (such as the SCC or the bacteriological analysis of milk samples) is required for an effective detection [12-14]. As a result, subclinical stages of mastitis are often undetected and have the greatest economic and sanitary consequences.

So far, several methods have been developed for the rapid diagnosis of subclinical mastitis. The SCC in milk is commonly used as a predictor of intramammary infection, and an elevated SCC in raw milk has a negative influence on its quality. The results of many studies [14-16] suggest that cows with SCC levels lower than 200,000 cells/mL have not been infected with major mastitis pathogens (namely, S. aureus and Streptococcus agalactiae), while cows with SCC above 300,000 cells/mL are probably infected. Alongside the false negatives, if the diagnosis is based on the SCC there is also the possibility of false positives. Sharma et al. (2011) reported that about 15% of milk samples from healthy cows is misclassified on the base of the SCC.

Moreover, the use of quarter milk samples for routine udder health monitoring has become expensive for large dairy herds, and initial udder health surveys are now conducted using composite cow milk samples [11]. Petzer, Karzis, Donkin, Webb and Etter [11] conducted a thorough study on the validity of somatic cell count as indicator of pathogen-specific intramammary infection which revealed that at a 200,000 cells/mL SCC threshold, the sensitivity for detecting major Gram-positive, Gram-negative and minor pathogens was 79.9%, 95.5% and 51.7% with specificity of 50.3%, 52.8% and 53.5%, respectively. At a 150 000 cells/mL SCC threshold, the sensitivity improved to 84.2%, 96.1% and 60.1% for the same microbial groups. Another study showed that the percentages of different pathogen species that were isolated from composite milk samples at a 150 000 cells/mL SCC threshold compared better than the percentages isolated at a 200 000 cells/mL threshold [17].

In order to overcome such limitations, efforts have been spent by scientists in search of suitable biomarkers for the rapid diagnosis of subclinical stages of cow mastitis [18-20]. In previous studies [21-24] it was found that total amino acid content increased significantly in mastitic milk compared to milk from healthy udders. In particular, the levels of branched chain amino acids (BCAAs) and some aromatic amino acids (AAAs) were found to be altered at subclinical stages. Moreover S. aureus

requires branched-chain amino acids (BCAAs: isoleucine, leucine, valine) for protein synthesis, branched-chain fatty acids synthesis and environmental adaptation. S. aureus needs to either synthesize BCAAs or scavenge them from the environment and it responds to their availability via transcriptional regulators. Increase of BCAAs in composite milk has been postulated to be linked to mammary infection by S. aureus [10,25-27]. Despite encoding the BCAA biosynthetic operon, S. aureus relies on the acquisition of BCAAs, most importantly leucine and valine, for rapid growth in media with excess or limiting concentrations of BCAAs, indicating that BCAA biosynthesis is typically repressed [28,29]. Paradoxically, biosynthesis remains repressed even in the absence of an exogenous source of leucine or valine, with growth of S. aureus observed only after a prolonged period, likely explaining why previous studies have been misled to conclude that S. aureus is auxotrophic for leucine and valine [26,30].

In an attempt to correlate the BCAAs isoleucine, leucine and valine and the aromatic amino acid tyrosine content in milk to S. aureus load, a new diagnostic tool was developed. For this purpose, in the present study, 65 composite milk samples during a S. aureus eradication intervention in a farm located in Umbria, central Italy, were submitted to direct HPLC analysis by ion-pairing reversed-phase method based on the use of the evaporative light-scattering detector (IP-RP-HPLC-ELSD) and analyzed for SCC and S. aureus content.

2. Materials and Methods

Milk sampling

All analyzes were performed on 65 samples collected at a dairy farm with known *S. aureus* problems. The dairy farm of choice is composed by 300 lactating Holstein Friesian cows and is located in Umbria region, central Italy. Average milk production is 30,2 Kg/day, all sampled cows were in their second or third lactation and between week-10 and weel-12 of lactation. Samples from composite quarter collection of individual cows were transported to the laboratory for SSC measurement and *S. aureus* isolation and identification and then frozen at -80 °C prior to further analysis.

The sample size was calculated using the formula n = Z2 * p * (1-p)/C2, where Z is the Z-value (e.g., 1.96 for a 95% confidence level), p is the expected prevalence, expressed as a decimal, and C is the confidence interval, expressed as a decimal [31]. With an expected prevalence of 50% (0.5), a confidence interval of 10 and a confidence level of 0.95 a sample size of 63 animals is then required. With 300 lactating cows in the farm, the sample size provides a 95% confidence level (CL) for cow-level prevalence, with a confidence interval (CI) of 10.

Somatic cells count

SCCs were measured at the APA (associazione provinciale allevatori) laboratory using a DeLaval cell counter according to the manufacturer's instruction (Cell counter DCC; DeLaval, Tumba, Sweden). 60 μL of sample were aspirated into a small cassette that contained a DNA-specific fluorescent reagent that bound to the SCC nuclei. The machine counted the fluorescent SCC nuclei in milk using an integrated digital camera.

S. aureus identification

The culture and identification of *S. aureus* isolates was done according to the methods described by Cenci Goga et. al (2003). Briefly, the first isolation medium was tryptose blood agar containing washed bovine red blood cells on which 1 mL aliquot of milk was spread and incubated at 37°C for 48 hours. Creamy, grayish-white or golden-yellow colonies, 3 to 5 mm in diameter, with a distinct zone of hemolysis, were considered to be presumptive *S. aureus*. These colonies were selected and tested for the following characteristics: cell morphology after Gram staining, for production of catalase, coagulase and for thermonuclease determination (TNase). Coagulase determination was performed using lyophilized rabbit plasma with EDTA (BBL Microbiology Systems, Cockeysville,

MD) reconstituted with sterile water. Suspect *Staphylococcus* spp. colonies were inoculated into 5 mL test tubes containing 0.5 mL of the reconstituted plasma. Clotting after incubation at 37°C between 4-24 hours was regarded as a positive result. TNase determination was performed according to the method described by Ibrahim [32]. After an 18-hour incubation at 37°C on Brain Heart Infusion (BHI) broth (Difco, BD Diagnostic Systems, MD), cultures were put into a water bath at 100°C for 15 minutes to eliminate any non-specific heat-labile nuclease activity, and centrifuged at room temperature for 30 minutes at 3000 rpm before testing. One hour before testing, 5 mm wells were made on plates containing Thermonuclease Agar with Toluidine Blue (Remel, Lenexa, KS). Approximately 70 µL of supernatant was then transferred into each well and the plates were incubated at 37°C for 4 hours. The presence of a pink halo surrounding the wells was regarded as a positive result. All Gram-positive cocci, catalase, coagulase positive and which produced TNase were identified as *S. aureus*.

Bacterial Strain and Artificial Specimen Preparation for Baseline Contamination

In order to quantify *S. aureus* load from the 65 frozen milk samples a procedure based on milk dilution followed by DNA extraction and nested PCR was adopted.

S. aureus ATCC 29213 was used to set up a baseline contamination level and adulterated milk specimens were prepared as follows. A *S. aureus* culture was grown in Nutrient Broth (NB, Oxoid CM0001, Basingstoke, UK) at 37 °C for 48 h on air. Total viable cells (TVC) count (on Nutrient Agar, NA, Oxoid CM0003, incubated at 37 °C on air for 24 hrs at 24 h was 1 x 10^8 CFU/mL. A 10mL aliquot of the culture was then added to 90 mL of raw milk to obtain a final concentration of 10^7 CFU/mL. Decimal dilutions were performed to obtain the following concentration in milk: 10^6 CFU/mL, 10^5 CFU/mL, 10^4 CFU/mL, 10^3 CFU/mL, 10^2 CFU/mL, 10 CFU/mL. TVC counts from all samples were recorded, as a control, on NA. Raw milk used had been previously tested for the absence of contaminating *Staphylococcus* spp.

DNA Extraction

Sixty-five milk samples after DNA extraction were tested by two-step PCR for the detection of *S. aureus*. Milk samples (1 mL), from both the real study cases and the previously prepared standard samples, containing *S. aureus* (0 - 10^6 CFU/mL) were each mixed with 1 mL phosphate-buffered saline (0.05% (v/v) Tween-20; PBST) vortexed and centrifuged at 10,000 *g* for 5 min. This procedure was performed to pellet the bacterial cells and to remove proteins and lipids in these milk samples, which may interfere with PCR amplification. Following the removal of the supernatant fluid, DNA was extracted using a sodium iodide (NaI) method [33]. The NaI extraction procedure was a modification of the method proposed by Ishizawa [34]. The pellet was re-suspended in 1 mL of TE buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA pH 8.0), transferred to a 2 mL microcentrifuge tube and added with 200 µL ammonium hydroxide, 200 µL absolute ethanol, 400 µL petroleum ether and 20 µL sodium dodecyl sulfate (SDS) 10%. The mixture was vortexed and then centrifuged at 15,000 *g* for 10 min. After supernatant removal, 1 mL of PBST was added and 100 µL transferred to a 2 mL microcentrifuge tube. A 300 µL volume of a solution containing 6 M NaI/13 mM EDTA/0.5% sodium N-lauroylsarcosine/10 µg glycogen/26 mM Tris-HCl, pH 8.0 were added to the tube, mixed and incubated at 60 °C for 15 min in a heating block. After addition of an equal volume of isopropanol the mixture was vigorously agitated and let stand for 15 min. The sample was then centrifuged at 10,000 *g* for 5 min to precipitate DNA and supernatant discarded. A 1 mL volume isopropanol 40 % was added and mixture vortexed. After centrifugation at 10,000 *g* for 5 min to recover DNA, the pellet was vacuum-dried. All operations were conducted at room temperature (RT). Extract DNA was stored at -20 °C prior to PCR.

Oligonucleotide Primers and DNA Amplification

All samples were tested to detect the presence of the *nuc* gene by means of PCR. To obtain very sensitive detection, a two-step PCR amplification procedure was developed with two nested sets of primers. In the first PCR, the primers *nuc_ext*-f (5′ GCGATTGATGGTGATACGGTT 3′) and *nuc_ext_int*-r (5′

GCCAAGCCTTGACGAACTAAAGC 3′), were used (MWG Biotech, Ebersberg, DE). The primers *nuc_e*-f and *nuc_ext_int*-r are located at positions 384 to 404 and 639 to 661, respectively, of the coding sequence (accession number NZ_LHUS02000183.1). By means of these primers, a fragment of 278 base pairs (bp) was amplified. For the nested PCR, the primer *nuc_i*-f (5′ AAAATGCAAAGAAAATTGAAGTC 3′) was used in combination with the same primer *nuc_ext_int*-r of the first PCR (5′ GCCAAGCCTTGACGAACTAAAGC 3′) (MWG Biotech). They are located at positions 152 to 174 and 256 to 278, respectively, of the amplicon obtained after the first PCR. The nested PCR amplified a DNA fragment of 127 bp. A 5 µL volume of each extracted sample was used for PCR which also contained 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 2.5 U of Taq DNA polymerase (Taq DNA Polymerase in Storage Buffer A, M1865, Promega Corporation, Madison, WI, USA), 0.1 mM of each appropriate primer, in a total volume of 25 µL. For the first PCR, 5 µL of sample DNA was added; for the second PCR, 5 µL of the first PCR product was used as template. DNA amplification reactions were carried out using a PCR Mastercycler (Eppendorf AG, Hamburg, DE) with the following programme. First PCR: denaturation at 95 °C for 3 min, 30 cycles each consisting of 1 min denaturation at 95 °C, 1 min annealing at 65 °C, 1 min extension at 72 °C and a final extension for 10 min at 72 °C: nested PCR: denaturation at 95 °C for 3 min, 30 cycles each consisting of 1 min denaturation at 95 °C, 1 min annealing at 54 °C, 1 min extension at 72 °C and a final extension for 10 min at 72 °C. In each PCR assay, a positive control with 100 ng of *S. aureus* ATCC 29213 DNA and a negative control without any bacterial DNA were included. A 10 µL aliquot of each PCR product was subjected to 1% (w/v) agarose gel electrophoresis containing 0.5 µg/mL ethidium bromide (Promega Corporation, M 5041) for 30 min at 100 V. PCR products (278 bp and 127 bp, respectively, for the first PCR and for the nested-PCR) were visualized under UV illumination. Their size was estimated using a standard DNA molecular weight marker (Novagen 69278-3, Madison, WI, USA).

For the 65 samples, 10-fold dilutions of milk were performed prior to DNA extraction and PCR results were recorded as the highest dilution still positive at the nested PCR. For the "artificial specimen preparation", *S. aureus* ATCC 29213 was cultured in NB and added to raw milk. Following plating of each dilution on NA, the numbers of CFU/mL were in accordance with expected values based on the decimal dilution factor. No *S. aureus* DNA was detectable in unadulterated milk. Bacterial DNA was successfully extracted from adulterated raw milk by NaI method. After the first PCR, the NaI method provided an upper detection limit of 10^6 CFU/mL but a not satisfying lowest limit. The second PCR with nested primers allowed a sensitive improvement in term of detection, thus allowing detecting a lowest limit of 10 CFU/mL. No visible amplicons were obtained from control samples where no *S. aureus* had been added.

HPLC Measurements and Extraction of free amino acids from the raw milk sample

Details on the procedure applied to extract the free amino acidic content from row milk samples have been reported elsewhere along with the experimental conditions for the IP-RP-HPLC-ELSD analyses [24].

Statistical methods

Statistical analyses were performed with the aid of StatView for Mac OS (SAS Institute, Inc. Cary, NC, USA). Pearson correlation and one-way ANOVA (Analysis of Variance) were used as a statistical test to assess the differences in means between the groups. Tukey-Kramer test at confidence level of 95%, was further employed for multiple comparisons between all pair-wise means to determine how they differ. $p < 0.005$ was considered statistically significant.

3. Results and Discussion

Free amino acidic forms were extracted from row milk samples, according to the procedure described elsewhere [24]. Before the IP-RP-HPLC-ELSD analyses, the extracts were lyophilized and then examined individually at a 20 mg/mL concentration. For the chromatographic assay, each

extract was dissolved in a hydro-alcoholic solution, and then eluted under the gradient conditions described by Ianni, Sechi, La Mantia, Pucciarini, Camaioni, Cenci Goga, Sardella and Natalini [24].

Results are summarized in Table 1. Prevalence of *S. aureus* ranged from 40,74% in milk samples with SCC < 100,000 to 87,5% in milk samples with SCC between 200,000 and 400,000. Isoleucine content ranged from 111 to 168 µg/mL, leucine from 126 to 250, valine from 226 to 428 and tyrosine from 97 to 127. *S. aureus* load ranged from 0.89 log cfu/mL (sd 1.37) in milk samples with SCC < 100,000 to 2.80 log cfu/mL (sd 1.64) in milk samples with SCC > 1,000,000. In order to find a relationship between *S. aureus* load associated to each milk sample and SCC and the concentration of Ile, Leu, Val and Tyr, first a bivariate Pearson correlation analysis was conducted (Figures 1 to 5). The correlation coefficient, *r*, was found to be 0.102 for SCC, 0.622 for isoleucine, 0.586 for leucine, 0.013 for valine and 0.07 for tyrosine, standing for a positive correlation between *S. aureus* and isoleucine and leucine concentration. No correlation was observed between *S. aureus* and SCC and between *S. aureus* and valine and tyrosine. Bar plot analysis (ANOVA and Tukey-Kramer test) (Figures 6 to 9) confirmed the differences in the amino acid content between the two groups of milk samples (*S. aureus* positive and *S. aureus* negative). This represents the first study in which a direct chromatographic method has revealed a statistically significant difference ($p<0.001$) of the content of isoleucine and leucine between *S. aureus* positive and *S. aureus* negative composite milk samples. On the other hand, the content of valine and tyrosine was not statistically different in the two groups ($p=0.761$ for valine and $p=0.354$ for tyrosine). This finding brings into question the use of SCC test as a survey tool to identify intramammary infections under field conditions, especially for eradication campaign. In fact, although numerous factors can influence the SCC at individual cow- and udder-quarter level, such as parity, lactation stage, incorrect milking machine settings, stress and other factors including genetics, the most important cause remains the infection status of the mammary gland [11]. Table 2 and 3 show sensitivity (*se*) and specificity (*sp*) calculated for SCC and for isoleucine and leucine content used as screening for *S. aureus* positive composite milk samples. From these data, along with data from Table 1, it appears that it is important to know how reliable SCCs of composite milk samples are as indicators of intramammary infection and *S. aureus* culture-positive results. Our data (Table 2) suggest that the best combination for *se* and *sp* is for a SCC threshold of 150,000 cells/mL (se 0.58; 0.41 – 0.75, sp 0.67; 0.51 – 0.83) and that the highest *se* is obtained when the threshold is lowered to 100,000 cells/mL (0.71; 0.57 – 0.85). High *se* is needed when surveying a herd for the purpose of identifying samples with positive bacterial growth. Our data (Table 3) prove also that, in the herd studied, isoleucine and leucine content, at threshold of 100 µg/mL, have always higher *se* and *sp* values than SCC. These results, in combination with data from previous studies of our research group [23,24], indicate that BCAAs isoleucine and leucine content are a promising marker of udder infection by *S. aureus*.

Table 1. SCC, *S. aureus* load and selected amino acids content for 65 composite milk samples.

	ssc count (x 1,000)		***S. aureus* +ve (n)**	*S. aureus* load (log cfu/mL)		Ile (µg/mL)		Leu (µg/mL)		Val (µg/mL)		Tyr (µg/mL)	
scc groups	**mean**	**sd**		mean	sd	mean	sd	mean	sd	mean	sd	mean	sd
< 100 (n = 27)	43.6	32.6	11	0.89	1.37	118	32	135	57	428	638	127	138
100-199 (n=15)	142.6	26.1	8	1.53	1,60	111	33	126	51	256	153	97	60
200-499 (n=8)	364.3	57.8	7	1.88	1,25	118	34	144	49	246	139	113	51
500-999 (n=10)	672.2	147.5	8	2.00	1,63	128	47	173	109	226	104	109	43
> 1,000 (n=5)	2,967.2	941.3	4	2.80	1,64	168	54	250	143	335	203	165	58
All (n=65)	427.5	808.9	38	1.48	1,54	122	38	149	79	330	433	118	97

Table 2. Sensitivity (se) and specificity (sp) with 95% lower (LCI) and upper (UCI) confidence interval for various SCC and isoleucine (Ile) and Leucine (Leu) threshold.

threshold	se	95% LCI and UCI	sp	95% LCI and UCI
SCC > 400,000/mL	0.37	(0.14 – 0.60)	0.89	(0.80 – 0.98)
SCC > 200,000/mL	0.50	(0.30 – 0.70)	0.85	(0.74 – 0.96)
SCC > 150,000/mL	0.58	(0.41 – 0.75)	0.67	(0.51 – 0.83)
SCC > 100,000/mL	0.71	(0.57 – 0.85)	0.59	(0.19 – 0.41)
Ile > 100 µg/mL	0.97	(0.93 – 1.00)	0.70	(0.50 – 0.90)
Ile > 150 µg/mL	0.32	(0.07 – 0.56)	0.93	(0.85 – 1.00)
Leu > 100 µg/mL	0.92	(0.84 – 1.00)	0.52	(0.28 – 0.76)
Leu > 100 µg/mL	0.55	(0.37 – 0.74)	0.78	(0.65 – 0.91)

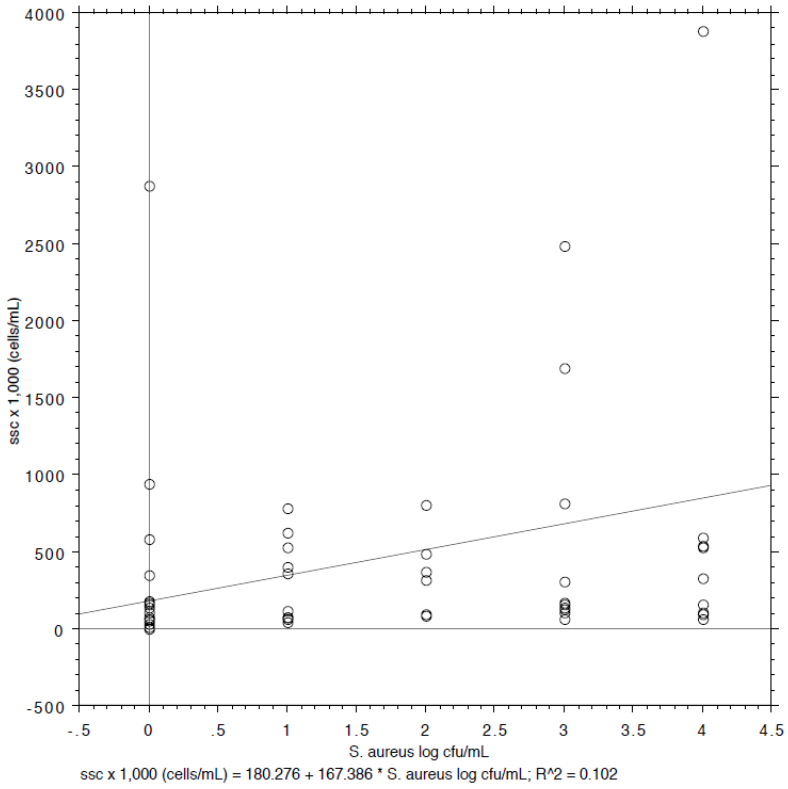
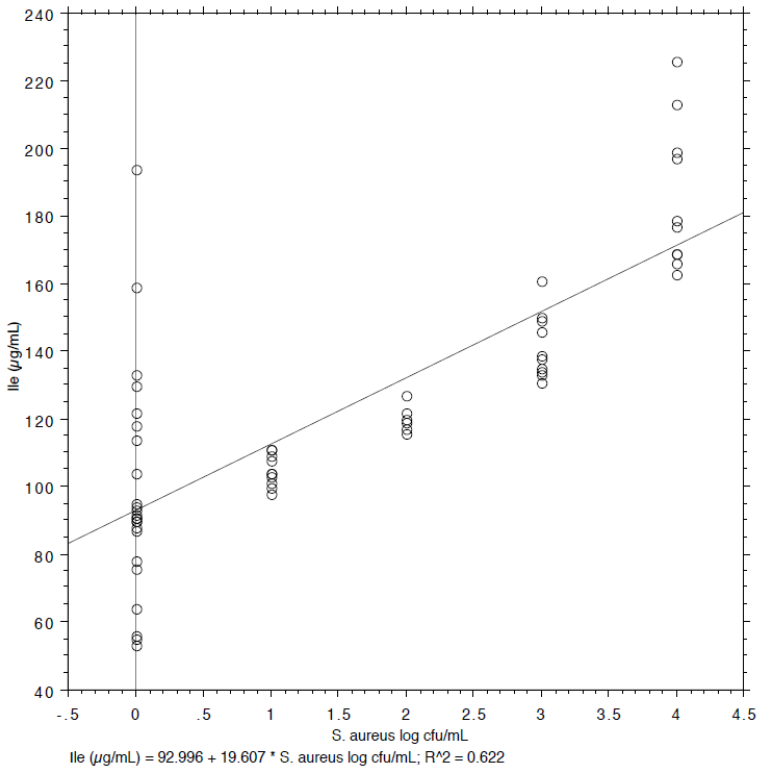


Figure 1. Pearson correlation between SCC and *S. aureus* load.



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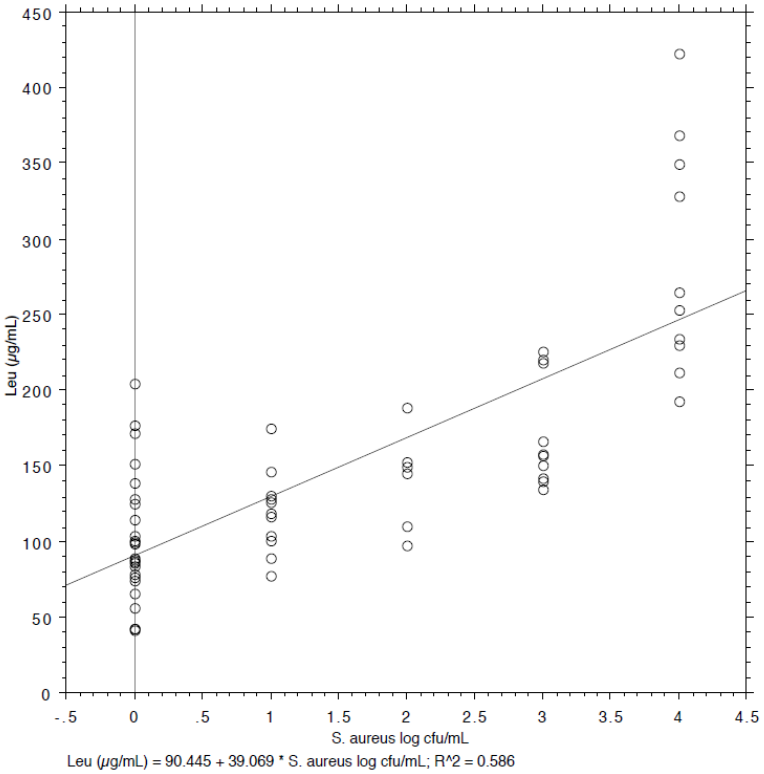
280 **Figure 2.** Pearson correlation between isoleucine content and *S. aureus* load.



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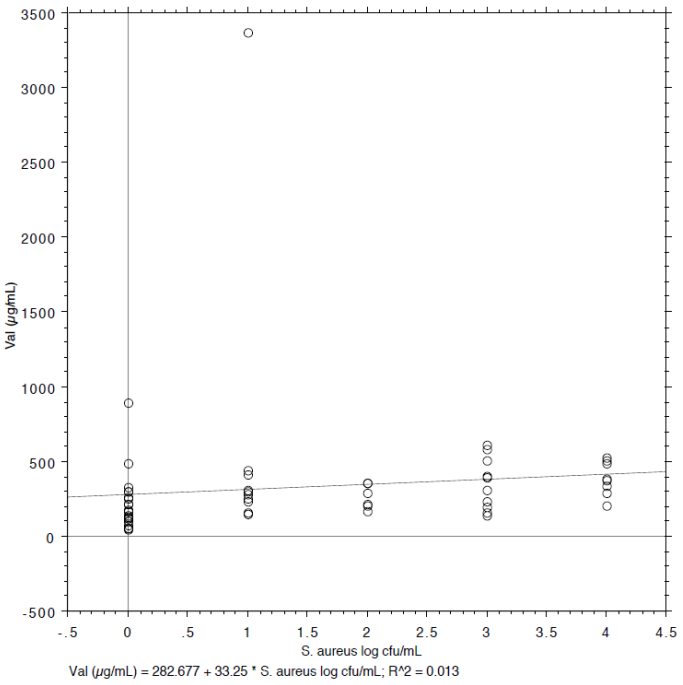
282 **Figure 3.** Pearson correlation between leucine content and *S. aureus* load.



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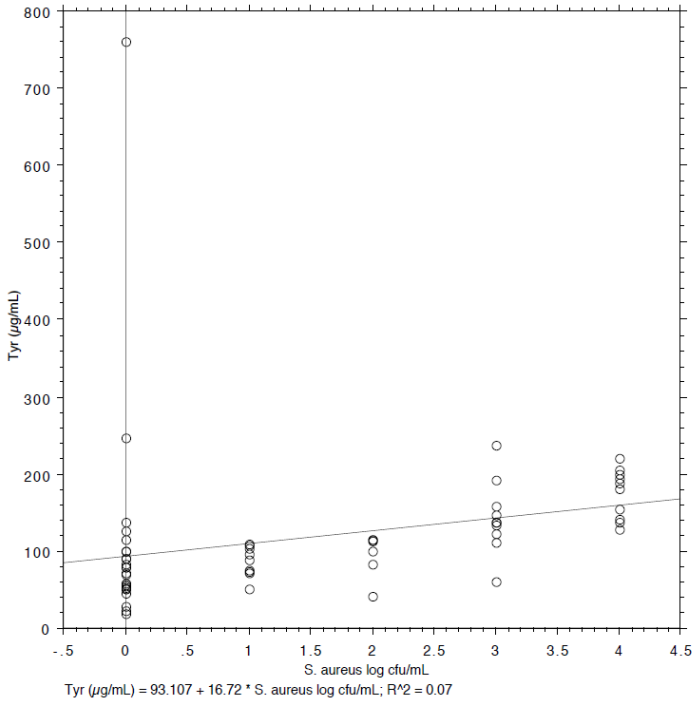
284 **Figure 4.** Pearson correlation between valine content and *S. aureus* load.



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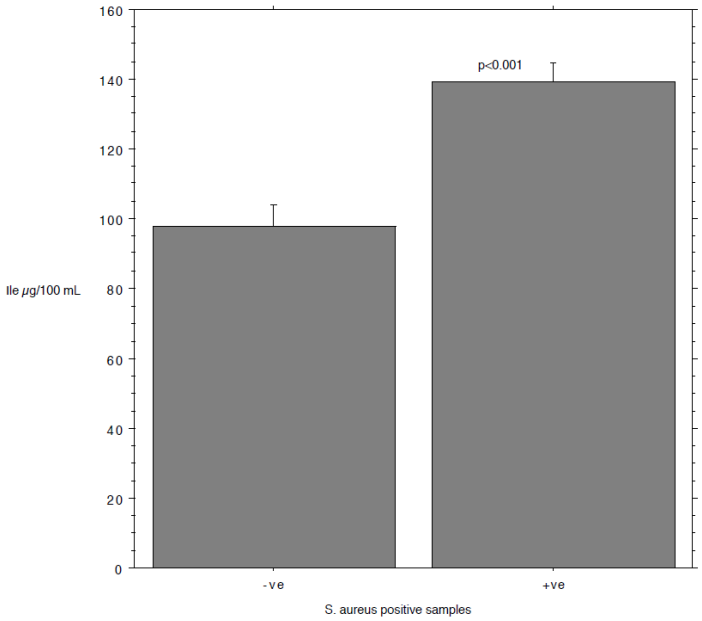
286 **Figure 5.** Pearson correlation between tyrosine content and *S. aureus* load.



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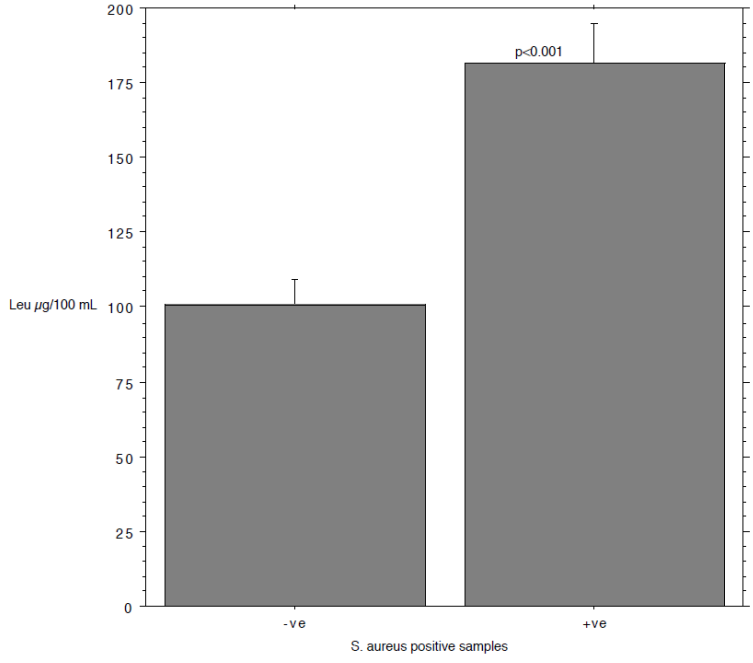
288 **Figure 6.** Bar plot for isoleucine content (mean and standard error) and ANOVA/Tukey-Kramer test
289 comparing *S. aureus* positive and negative samples.



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291 **Figure 7.** Bar plot for leucine content (mean and standard error) and ANOVA/Tukey-Kramer test
292 comparing *S. aureus* positive and negative samples.

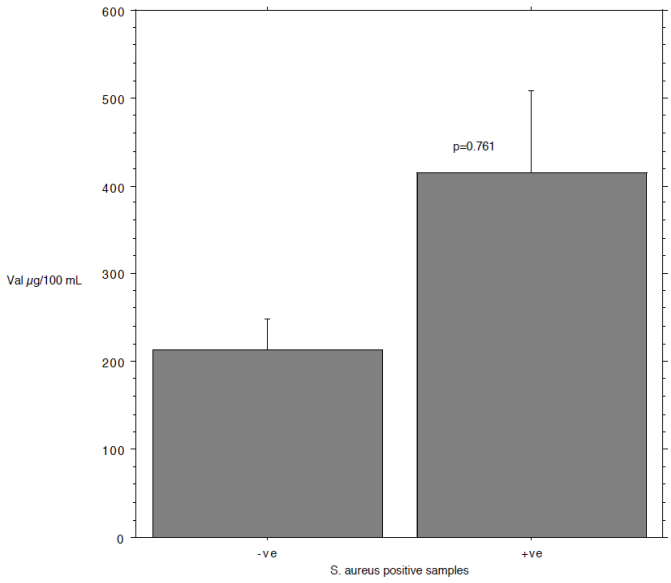

Figure 8. Bar plot for valine content (mean and standard error) and ANOVA/Tukey-Kramer test comparing *S. aureus* positive and negative samples.

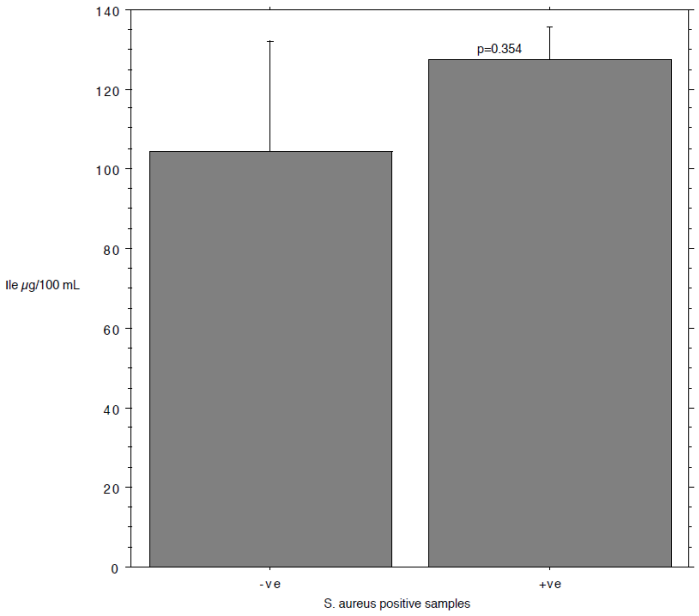

Figure 9. Bar plot for tyrosine content (mean and standard error) and ANOVA/Tukey-Kramer test comparing *S. aureus* positive and negative samples.

5. Conclusions

S. aureus mastitis comes with widespread metabolic perturbations and the BCAAs appear to be among the most distinctly perturbed metabolites. In the present work, an ion-pairing reversed-phase method based on the use of the evaporative light-scattering detector and on the use of the heptafluorobutyric acid as the ion paring agent was successfully applied for the direct analysis of the amino acid content in 65 milk samples. The application of the ANOVA and Tukey-Kramer analysis shed light on statistically significant differences in the content of the BCAAs isoleucine and leucine, between the two groups based on *S. aureus* positivity ($p<0.001$), while bivariate Pearson correlation analysis showed a strong relationship between *S. aureus* load and the content of these two BCAAs. Grounded on these evidences, it is clear that there could be a great deal of clinical value in monitoring

isoleucine and leucine levels in milk, given also the low correlation between SCC and *S. aureus*. Although the use of BCAAs as biomarkers of *S. aureus* infection has great potential, questions remain regarding the biochemical mechanisms underlying the relationship of BCAAs to disease process and severity.

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