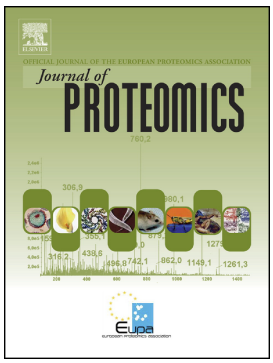

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Influence of subclinical mastitis and intramammary infection by coagulase-negative staphylococci on the cow milk peptidome

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Short title: **Milk peptidomics in CNS mastitis**

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Highlights

- detailed characterization of the milk peptidome in subclinical CNS mastitis
- very large milk peptide dataset available for healthy and mastitic milk
- demonstration that subclinical CNS mastitis impacts significantly on the milk peptidome
- demonstration of the direct relationship with the SCC

Abstract

Coagulase-negative staphylococci (CNS) are the most prevalent microorganisms isolated from cow milk and are associated with subclinical mastitis and persistent increases in the bulk milk somatic cell count (BMSCC) of low BMSCC herds. By combining peptide enrichment, LC-ESI-MS/MS, and statistical analysis, we investigated the influence of subclinical mastitis and CNS infection on the milk peptidome. Quarter milk samples from clinically healthy Holstein cows were subjected to bacteriological culture (BC) and somatic cell counting (SCC) for two consecutive samplings and 28 (including 11 negatives and 17 positives) were selected for peptidomic analysis.

The study identified 1363 different endogenous peptides and highlighted a significant increase of peptides in CNS-positive milk, mainly represented by casein fragments. Milk peptidome changes increased with the SCC, as also demonstrated by protein electrophoresis and densitometry. Peptides significantly different in CNS or CONTROL samples were identified and characterized. Our results indicate that subclinical mastitis by CNS can induce significant changes in the milk peptidome, opening the way to future studies for the identification of a biomarker panel as well as for the understanding of their consequences for the technological and sensorial characteristics of cow milk and dairy products.

Keywords:

Subclinical mastitis; coagulase-negative staphylococci; milk; peptidomics; tandem mass spectrometry.

1. Introduction

Intramammary infections (IMI) and mastitis are among the most relevant health issues in dairy cattle [1,2]. IMI agents include a variety of gram-positive and gram-negative microorganisms, ranging from contagious pathogens such as *Staphylococcus aureus* and *Streptococcus agalactiae* to several environmental organisms of varying virulence including *Streptococcus* spp., *Enterococcus* spp., *Corynebacterium* spp., *Micrococcus* spp., to the Gram-negatives *Escherichia coli*, *Klebsiella* spp., and several other environmental pathogens, including algae and fungi [3]. Coagulase-negative staphylococci (CNS) species, recently defined as non-aureus staphylococci (NAS) when identified with methods different from the standard coagulase test [4], have long been considered as harmless udder colonizers [5]. In the last twenty years, however, CNS have become the most prevalent pathogens in dairy cows worldwide, especially in Europe and in the US [6–10] with figures ranging from 27% to 55% [11,12]. The detection of CNS as the most frequently isolated bacteria in bovine milk has been related to the widespread adoption of suitable farm and animal management practices enabling the successful control of contagious pathogens and the more obvious environmental microorganisms, bringing CNS into focus [9,13–15] for their ability to cause persistent IMI generally accompanied by increased somatic cell count (SCC) and reduced milk production [10]. Although a protective effect against clinical mastitis has been postulated [16], according to most authors CNS IMI are responsible for a large proportion of bulk milk SCC (BMSCC) increases in low BMSCC herds [4].

Clinical mastitis leads to important economic losses due to animal culling, discarded milk, and reductions in milk yield and quality [17,18], but subclinical infections also represent a relevant issue due to recurrent cases, to the absence of clinical signs, and the consequent difficult diagnosis and sometimes control, required for avoiding increases in the BMSCC with the related milk quality losses [1]. Clinical mastitis is characterized by an increase in temperature, swelling of the mammary gland, redness, and pain at palpation, and milk shows several alterations including the presence of clots and high SCC [19]. On the other hand, subclinical mastitis is typically diagnosed by detecting an increase in milk somatic cells either with dedicated instruments or with the California Mastitis Test (CMT), a widespread cow-side test [20]. The value of the SCC in diagnosing mastitis is based on the migration of leukocytes from the circulation and into

the milk occurring as a result of the inflammatory process elicited by pathogenic bacteria [21]. In healthy cow milk, SCC is normally below 100,000 cells/mL, and a conventional threshold of 200,000 cells/mL is applied for ensuring an adequate specificity of mastitis detection [22]. In the infected mammary gland, neutrophils and macrophages phagocytize bacteria and release significant amounts of proteases and other cellular contents into the milk [23–26], leading to compositional changes both in terms of proteins and of peptides produced as a result of the proteolytic activity exerted by endogenous enzymes on abundant milk proteins [21]. Besides, infecting bacteria may release exogenous enzymes contributing to proteolysis [27]. Accordingly, the milk proteome and peptidome are known to change with mastitis [27–31].

Previous studies addressing the peptidomic changes occurring in cow milk during mastitis have focused mainly on clinical mastitis, either spontaneous [31] or induced by experimental infection [29]. Mansor and coworkers [31] were the first to use mass spectrometry to demonstrate that several peptides increased in milk from cows with clinical *S. aureus* or *E. coli* mastitis were mainly derived from Alpha-S1 and Beta-casein. In that study, 48 peptides were significantly different between the milk of healthy and mastitic cows. Thomas and coworkers [29] expanded the peptidomic repertoire in a study evaluating the kinetics of experimental *S. uberis* infection and found signature peptides with potential as mastitis markers. Only one study evaluated the milk peptidome in subclinical mastitis, demonstrating that even subclinical infections can cause significant increases in the total number of released peptides when compared to uninfected milk [27]. However, neither the IMI agents nor the SCC was reported. In this study, we focused on the changes occurring in the milk peptidome during subclinical CNS infection and mastitis. To this aim, we applied a peptide enrichment protocol and a high-performance liquid chromatography/tandem mass spectrometry analytical approach followed by bioinformatic analysis.

2. Materials and methods

2.1. Animals and milk samples

Milk samples were collected in a commercial dairy farm with an average of 150 Holstein milking cows housed in free stall barns in deep-bedded cubicles with straw, located in the Bergamo area (Italy). All cows

were fed with a balanced TMR in feed alleys with headlocks. Lactating cows were milked twice a day in a double-10 herringbone milking parlor. Quarter milk samples were collected from 147 lactating cows according to the NMC guidelines for assessing udder health [32]. Cows were sampled twice one week apart. Briefly, all teats were carefully cleaned before sampling with a pre-dipping foam containing lactic acid and the apex was disinfected with alcohol. The first three streams of foremilk were discarded and then approximately 10 mL of milk was collected aseptically from each teat into sterile vials. Samples were stored shortly at 4°C until bacteriological assays and SCC tests were carried out.

2.2. Somatic cell counting, bacteriological analysis, and milk sample selection

Bacteriological analysis was carried out according to the NMC guidelines [32]. Ten µl of milk was seeded on blood agar and incubated aerobically at 37°C. Colonies were observed after 24 h of aerobic incubation and provisionally identified based on morphology and haemolysis patterns. Gram-positive cocci were evaluated for the presence of reactions to the catalase (positive) and coagulase (negative) test for classification as coagulase-negative staphylococci (CNS). The SCC was evaluated with a Bentley Somacount 150 (Bentley Instrument, Inc., Chaska, MN, USA). Milk samples from quarters that were CNS-positive for two consecutive samplings were included in the study as CNS, while milk samples from quarters that remained negative to bacteriological culture and had very low SCC for two consecutive samplings were included as Controls. Details on sampled cows, including quarter, parity, days in milk (DIM), and SCC at the first and second sampling are provided in **Table S1**.

2.3. Sample preparation for shotgun peptidomics

Milk was defatted by centrifugation at 1220x g x 4 min at room temperature. Then, 500 µL of defatted milk from CNS (n=17) and Control cows (n=11) were diluted with an equal volume of 32% (v/v) acetic acid and ultra-filtered using Amicon Ultra-0.5 mL centrifugal filters (MWCO 10K) for high molecular weight protein depletion [33]. The filtrate was then precipitated with 2 volumes of cold acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA) and centrifuged at 13,200 rpm for 30 minutes to remove residual proteins. The

supernatant was collected, dried, dissolved in 1% (v/v) formic acid and desalted (Zip-Tip C18, Millipore) before mass spectrometric (MS) analysis [34].

2.4. LC-ESI-MS/MS analysis

NanoHPLC coupled to MS/MS analysis was performed on Dionex UltiMate 3000 directly connected to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) by a nanoelectrospray ion source. Peptide mixtures were enriched on 75 µm ID x 150 mm Acclaim PepMap RSLC C18 column and separated employing the LC gradient: 4% ACN in 0.1% formic acid for 3 min, 4–28% ACN in 0.1% formic acid for 130 min, 28–40% ACN in 0.1% formic acid for 20 min, 40–95% ACN in 0.1% formic for 2 min and 95–4% ACN in 0.1% formic acid for 3 min at a flow rate of 0.3 µl/min. MS spectra of eluting peptides were collected over an m/z range of 375-1500 using a resolution setting of 120,000, operating in the data-dependent mode to automatically alternate between Orbitrap-MS and Orbitrap-MS/MS acquisition. HCD MS/MS spectra were collected for the 20 most abundant ions in each MS scan using a normalized collision energy of 30%, and an isolation window of 1.7 m/z. Rejection of +1, and unassigned charge states were enabled.

2.5. Database search and peptide identification

Raw label-free MS/MS files from Thermo Xcalibur software (version 4.1) [35] were analyzed using Proteome Discoverer software (version 2.2, Thermo Fisher Scientific) and searched with the Sequest algorithm against the proteome of *Bos taurus* from Uniprot 10-02-2019. Only peptides with high FDR confidence were considered (FDR 0.01 strict, FDR 0.05 relaxed) to remove false-positive matches. The assigned peptides are filtered by minimal peptide length (6 amino acid) and m/z accuracy (8 ppm). The quality of a match between sequence and observed peaks was provided by a high cross correlation score (≥ 1.5). PSM confidence was set to High.

Unspecific digestion was chosen, and neither fixed nor variable modifications were set. Resulting peptides and protein hits were further screened accepting only those hits listed as high confidence and with an Xcorr

≥ 1.5. Two replicates were carried out for each sample. Since a threshold must always be indicated in the statistical analysis of the output from a shotgun analysis for peptides identification, we set a cutoff of 70% similar to the one applied in Thomas et al, 2016 [29] who carried out a peptidomic investigation of milk from an experimental model of *Streptococcus uberis* mastitis in dairy cows. Peptides were considered increased or decreased if they showed a significant Welch t-test difference (cut-off at 1% permutation-based FDR) or if they were present with a frequency ≥70% in either the CNS or Control group but less than 70 % in the other group [36]. Peptide sequences were analyzed manually for C-terminal amino acids. The potential proteases generating the cuts were classified based on the MEROPS database [37] by evaluating the specificities of the main proteases generating the cuts [26].

Statistical analyses and PCA were performed using the Perseus software (version 1.5.5.3, www.biochem.mpg.de/mann/tools/). PCA was carried out first by considering each milk sample separately, and then after grouping and averaging quantitative data related to peptides in three sample groups as follows: Control, including all data from culture-negative samples, CNS, including all data from CNS-positive samples with SCC < 300,000 cells/mL, and CNS high SCC, including all data from CNS-positive samples with SCC > 300,000 cells/mL.

2.6. SDS-PAGE and densitometry

SDS-PAGE was carried out on home-made 12.5% gels on a Bio-Rad system (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer instructions as described previously [38]. Briefly, 0.5 µl of whole milk was mixed with loading buffer, denatured and reduced, loaded into the wells, and subjected to electrophoretic separation. The gels were stained with Coomassie for protein visualisation and digitalized with a Gel Doc System (Bio-Rad) for densitometric analysis. Densitometry was carried out with ImageJ [39] as follows. After automatic lane detection, background was subtracted with the rolling ball feature at 80% setting. Band volumes were normalized to the total lane volume. For analysis, six main areas of the gel were defined as follows, by referring to the gel area or the main protein component: Top MW (above 100 kDa); high MW (100-50 kDa); caseins (50-20 kDa); Beta-lactoglobulin (20-15 kDa); Alpha-lactalbumin (15-12

kDa); and low MW (below 12 kDa). Protein identities were derived from the literature [29]. Normalized band volumes calculated for each milk sample and falling within each gel area were then processed with Prism 8.3.1. for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com) for descriptive statistics and for evaluating the significance of the differences between classes based on the unpaired T-test.

2.7. Data Availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository [40] with the dataset identifier PXD017768.

3. Results

3.1. Common and differential peptides identified in Control and CNS milk

A shotgun label-free quantitative peptidomic approach was applied to investigate the milk peptidome of cows with subclinical CNS IMI when compared to healthy controls. The workflow included peptide enrichment by ultrafiltration, protein precipitation, and centrifugation, followed by LC-ESI-MS/MS analysis, identification, and label-free quantitation.

When considering as positive v identified all the endogenous peptides present in at least 70% of each data set, 1112 and 1300 peptides were detected in control and CNS milk, respectively, for a total of 1363 identified peptides. Of these, 251 were present with a frequency ≥ 70% in CNS and < 70% in Control, 63 were present with a frequency ≥ 70% in Control and < 70% in CNS, and 1049 were present in both CNS and Control (**Figure 1A**). Among the 1049 peptides identified in both groups, 112 were increased and 89 were decreased in CNS in comparison to Control milk (Welch's t-test: FDR 0.01). The corresponding Volcano Plot is reported in **Figure 1B.** Overall, the analysis identified 363 peptides (from now INCREASED in CNS) which were increased (112) or present in at least 70% of CNS milk (251) (**Table S2**), and 152 peptides (from now DECREASED in CNS) which were decreased (89) or present in at least 70% of Control milk (63) (**Table S3**). In

Tables S2 and **S3**, all the peptides with 100% sequence identity with peptides previously identified in milk during mastitis [27, 29, 31] are indicated with an "X". The 515 differential peptides ranged in length from 8 to 64 amino acids with an average length of 22, with molecular masses mainly in the range 1200–2000 Da. All the identified peptides originated from the 112 proteins listed in **Table S4** and reported in **Figure 2**. Of these, 80% of the peptides common to CNS and Control milk were produced by the proteolysis of 11 milk proteins, namely: Alpha-S1-casein, Alpha-S2-casein, Beta-casein, Butyrophilin subfamily 1 member A1 (BTN1A1), Lactoperoxidase, Glycosylation-dependent cell adhesion molecule (GLYCAM-1), PIGR protein, Sodium-dependent phosphate transport protein 2B, Glycoprotein 2, Beta-lactoglobulin, Serum amyloid A protein (**Figure 2A**). Fewer peptides were derived from Kappa-casein, Fibroblast growth factor-binding protein 1, Beta-1,4-galactosyltransferase 1, Complement component C3, Perilipin, Cysteine-rich secretory protein 2, Mucin-15, Parathyroid hormone-related protein, Beta-1,3-N-acetylglucosaminyltransferase lunatic fringe, FXYD domain-containing ion transport regulator, as shown in **Figure 2A**.

On the other hand, **Figure 2B** illustrates the proteins originating the differential peptides observed in the comparison Control *vs* CNS. In the latter case, the most represented proteins were Alpha-S1-casein (116 peptides increased *vs* 9 peptides decreased in CNS), Alpha-S2-casein (65 peptides increased *vs* 1 peptide decreased in CNS), Beta-casein (107 peptides increased *vs* 14 peptides decreased in CNS) and Kappa-casein (13 peptides increased *vs* 1 peptide decreased in CNS). DECREASED peptides in CNS were mostly derived by GLYCAM1 and BTN1A1 with 19 peptides and 17 peptides decreased in CNS milk, respectively.

Figure 3A reports the PCA carried out on the peptidome of all samples. While the analysis did not show differential clustering between control and CNS, it clearly differentiated SCC samples with very high SCC counts (28CNS, 29 CNS and 32 CNS, Table S1). This was more evident when reporting the analysis obtained after grouping and averaging quantitative data related to peptides in Control, CNS with SCC < 300,000 cells/mL, and CNS with SCC >300,000 cells/ mL (**Figure 3B**). Therefore, three subgroups were identified and their peptide profiles were compared: Subgroup 1 (Neg, SCC < 10,000 cells/mL, N = 11), Subgroup 2 (CNS, SCC > 100,000 cells/mL, N = 12) and Subgroup 3 (CNS, SCC < 100,000 cells/mL, N = 5), based on the sample characteristics reported in **Table S1**.

3.2. Differential analysis on subgroups: Subgroup 1 (Neg, SCC < 10,000 cells/mL) vs Subgroup *2 (CNS, SCC > 100,000 cells/mL)*

Upon comparison of Subgroup 1 (Neg, SCC < 10,000 cells/mL) *vs* Subgroup 2 (CNS, SCC > 100,000 cells/mL), 1128 and 1352 peptides were detected in at least 70% of the samples of Subgroup 1 and Subgroup 2, respectively; among them, 1046 peptides were identified in both groups, 82 peptides with a frequency ≥ 70% in Subgroup 1 and < 70% in Subgroup 2, 306 were present with a frequency ≥ 70% in Subgroup 2 and < 70% in Subgroup 1.

Using a Welch's t-test (FDR 0.01) to identify differentially abundant peptides, 179 peptides were increased and 111 were decreased in Subgroup 2. Therefore, this latter analysis identified 485 peptides which were INCREASED in Subgroup 2 (179 increased + 306 with a frequency ≥ 70% in Subgroup 2 and < 70% in Subgroup 1) (**Table S5**) while 193 peptides were DECREASED in Subgroup 2 (111 decreased + 82 with a frequency ≥ 70% in Subgroup 1 and < 70% in Subgroup 2. (**Table S6**).

3.3. Differential analysis on subgroups: Subgroup 2 (CNS, SCC > 100,000 cells/mL) vs Subgroup 3 (CNS, SCC < 100,000 cells/mL)

To assess the impact of SCC on the peptidome of CNS-positive milk, we compared the 306 peptides detected in Subgroup 2 with a frequency ≥ 70% (originated from the previous analysis between Subgroup 2 vs Subgroup 1) with the total peptides detected in Subgroup 3, characterized by the presence of CNS without detectable increase in SCC. Among the 306 peptides identified in Subgroup 2, 48 peptides were found in Subgroup 2 and not in Subgroup 3 (**Table S7)** and originated from the proteolysis of 9 milk proteins, namely Alpha-S1 casein, Alpha-S2-casein, Beta-casein, Kappa-casein, GLYCAM-1, Mucin-15, 40S ribosomal protein S21, Serum amyloid A protein and Osteopontin. Of these, only three peptides belonging to caseins had been reported previously, and only in subclinical mastitis [27].

3.4. Impact of CNS IMI and SCC on high-abundance milk proteins

The influence of subclinical CNS IMI and increased SCC on high-abundance milk proteins was then investigated also by SDS-PAGE and densitometry. The results are summarized in **Figure 4**. **Figure 4A** reports the representative SDS-PAGE pattern of milk proteins in Control milk and CNS milk. Protein profiles were compared i) by considering the bacteriological status alone (**Figure 4B**, CNS-positive *vs* Control) and ii) by considering the bacteriological status and two SCC thresholds of 100,000 and 200,000 cells/mL. Results are illustrated in **Figure 4C** and **Figure 4D**, respectively.

When samples were classified only according to their CNS status, the differences between the two classes were not statistically significant, although some trends indicating an increase in high MW and low MW components and a decrease in caseins were observed. When considering CNS-positive milk with SCC > 100,000 cells/mL (Figure 1C), the low MW component was significantly increased in comparison to all the other samples. In CNS-positive milk with > 200,000 cells/mL, statistically significant increases were observed also for high MW proteins, mainly represented by lactoferrin, bovine serum albumin, and immunoglobulin heavy chains, accompanied by a statistically significant decrease in caseins.

3.5. Peptide classification and protease prediction

The differential peptides were manually analyzed and classified according to their C-terminal amino acid. As illustrated in Figure 5, R at the C-term was considerably less frequent in the peptides unique or higher in healthy milk. On the other hand, other amino acids, except for L, H, and N, were more frequent at the C-term of CNS-positive milk.

4. Discussion

This work assessed the peptidomic changes occurring in the milk of cows with subclinical CNS IMI by applying a highly sensitive analytical pipeline based on peptide enrichment and characterization by high-performance tandem MS. This led to the generation of a very large peptide dataset available for both healthy [41] and mastitic cow milk [29].

When comparing the CNS and Control milk peptidomes, we observed a significantly higher number of endogenous peptides in CNS milk. This indicated a higher proteolytic activity, in agreement with the only milk peptidomic investigation currently available for subclinical mastitis [27] and confirmed that subclinical CNS IMI has significant consequences on the peptidome adding to those already reported for the BMSCC, even in the absence of clinical signs [14]. Also in line with the data reported for clinical mastitis [29,31], caseins were the proteins most affected by this increased proteolysis. Along with caseins, serum amyloid A protein (SAA3) was also extensively digested in CNS-positive milk, in keeping with previous reports in acute mastitis and with the indication of SAA3 as a mastitis biomarker in cows [42]. Interestingly, peptides derived from both bovine alpha-S1-casein and human M-SAA3 were found to be related to immune defences of the mammary gland. At least four alpha-S1-casein-derived antimicrobial peptides were found as having antibacterial activity against pathogens such as *Enterobacter sakazakii* and *Escherichia coli* [43]. Three of these peptides, namely IKHQGLPQE and VLNENLLR (also called Caseicin A and B), were found in the present study as part of alpha-S1-casein longer peptides present in CNS milk, whereas Isracidin (RPKHPIKHQGLPQEVLNENLLRF) was found as a full peptide. A fourth peptide, namely SDIPNPIGSENSEK (Caseicin C), was identified in control milk. Peptides from human M-SAA3 were also found to have a protective activity on intestinal epithelial cells against enteropathogenic *Escherichia coli* (EPEC) adherence, although in vitro results were not confirmed by *in vivo* studies [44], although none of the M-SAA3 sequences with anti-bacterial activity was found in the present study. This result is remarkable because demonstrated for the first time the capability of CNS of generating peptides with anti-microbial activities, at least from alpha-S1-casein.

On the other hand, some peptides were less represented in CNS-positive milk and were derived mainly from GLYCAM-1, also known as lactophorin or proteose peptone 3 (PP3), and BTNA1. GLYCAM-1 has been implicated in the pathogenesis of *S. uberis* mastitis [45], and a decrease in BTN1A1 has been reported in the milk from cows with mastitis [46,47]. Both GLYCAM-1 and BTN1A1 are abundant components of the milk fat globule membrane and play an important role in the physiology of milk production and the fat globule secretion process [48–50]; this finding might be related to the impairment of physiological mammary gland

functions during mastitis [21]. However, the higher abundance of GLYCAM-1 peptides in Control milk is in contrast with the findings of Guerrero and coworkers [27]. The number of peptides originating from Beta-lactoglobulin was lower in CNS (no peptides increased *vs* 4 peptides decreased in CNS) suggesting that this high-abundance protein may also be reduced during subclinical mastitis, in agreement with previous reports [28].

Even if the number of identified peptides is the highest reported so far in mastitic milk, very few peptides originating from serum albumin, lactotransferrin, and immunoglobulin heavy or light chains were detected, and none of these increased or decreased significantly as a result of CNS IMI. However, this might be due to their higher resistance to proteolysis while caseins might be more sensitive, because of their different respective roles in host defense and offspring physiology and nutrition. Moreover, no peptides derived from cathelicidins nor haptoglobin were detected in CNS milk, despite the numerous reports indicating these proteins as the most valuable mastitis biomarkers in different dairy ruminant species [51–55]. However, this is also in line with previous studies as no peptides resulting from their degradation have been detected so far in subclinical or clinical mastitis. In this case, also, the reason might reside in higher resistance to proteolysis in comparison to caseins, as postulated above. Furthermore, given the background that the experimental model designed for the present study included milk from subclinical mastitis, limited plasma exudation through the entero-mammary barrier is expected. Local production from inflamed mammary gland cells, the other potential source of acute phase proteins, is also supposed to be reduced, and likely limited to M-SAA3 that is regarded as one of the major reactants in mammary gland epithelium [56].

The peptidomic results, together with the behavior of abundant milk proteins, supported a direct relationship of SCC with proteolysis: i) the PCA sample clustering based on all identified peptides highlights an influence of the SCC; ii) the number of INCREASED peptides is higher in CNS samples with SCC > 100,000 cells/mL; iii) the densitometric analysis of proteins highlights the significant decrease in caseins and increase in low MW and high MW components with increasing SCC; iv) casein-derived peptides increase; and v) 48 peptides are unique to CNS milk with increased SCC. Furthermore, the distribution of differential

peptides based on their C-terminal aminoacid showed a strikingly higher frequency of peptides ending with R in healthy milk as opposite to peptides ending with other peptides (especially V, F, and A) in CNS-positive milk. According to the MEROPS database, plasmin generates peptides ending with R at the C-term, while elastase, cathepsin D, cathepsin B, cathepsin G, MMPs 2, and MMPS 9 do not generate peptides ending with R but do generate peptides ending with V, F, and A at their C-term [37]. Plasmin is the principal proteinase in healthy milk, while other proteinases including elastase, cathepsins, and metalloproteases, are active particularly as the SCC of milk increases [26], having mainly polymorphonuclear neutrophil granulocytes as a source. Taken together, this might be the result of a more intense proteolytic activity by endogenous proteases released by inflammatory cells in milk [27–29,57] and indicates that even minimal SCC changes induced by CNS IMI influence the milk protein and peptide abundances, with possible negative consequences on the milk sensorial and technological properties [58].

According to PCA analysis of peptidomic data, however, samples did not cluster according to CNS positivity. Rather, a clear separation was observed only with CNS showing the largest SCC increase. Concerning control sample 9, that clustered with high SCC, CNS positive samples, it should be noted that it is the only control sample in advanced lactation (over 300 DIM) while all the other control samples were around or below 100 DIM (Table **S1**). It is long known that the stage of lactation and lactation number can influence the endogenous proteolytic activity of milk, which is highest when approaching the dry period [59,60]. This might be the reason for the different behavior of this control sample. Therefore, when looking for mastitis-specific peptides, the impact of parity and stage of lactation should also be considered.

The peptides specifically identified in subgroup 2 (CNS positive, SCC > 100,000 cells/mL) combine presence of CNS with increased SCC. As mentioned above, numerous peptides belonging to SAA were identified. Most were present also in Control milk, but two were detected with a frequency ≥ 70% in subgroup 2 and might deserve further investigation. Peptides originating from Osteopontin were also present in subgroup 2 with a frequency ≥ 70%. Osteopontin acts as a cytokine involved in enhancing the production of interferon-gamma and interleukin-12 and reducing the production of interleukin-10 and is essential in the pathway that leads to type I immunity [61]. Peptides belonging to this protein have also been reported in subclinical

mastitis by Guerrero *et al.*, although not in all milk samples examined [27]. Nevertheless, these authors did not report the etiological agents of IMI that might belong to different microbial species. Osteopontin peptides were not detected by Thomas *et al.* in experimental *S. uberis* clinical mastitis [29] and might be produces only during subclinical mastitis.

On the other hand, several peptides were detected higher frequency in healthy milk. This might be due to the higher integrity of caseins in these samples, and therefore to the reduced load of casein-derived peptides that increase sample diversity enabling the detection of less abundant peptide species. Accordingly, peptides with higher frequency in healthy milk were mostly derived from lower abundance proteins involved in physiological processes. Among them several originated from proteins implicated in fatty acid metabolism and mitochondrial components (Table S6) like CD109 and CD14. CD109 is a B-lymphocyte antigen, while CD14 is a monocyte/macrophage marker. This is in line with the prevalence of macrophages and lymphocytes in healthy milk, as opposite to neutrophils that predominate in mastitic milk [21]. These peptides might be of interest for monitoring changes in milk cell composition and the onset of inflammation.

Specific and thoroughly validated peptide marker panels, including approaches based on targeted MS approaches carried out on larger sample cohorts with the identification of CNS also at the species level, would undoubtedly be valuable for identifying and characterizing mastitis due to different IMI agents. However, the implementation of peptides as biomarkers presents numerous challenges. Advanced analytical methods and sophisticated instrumentation are needed for their detection and identification. Besides, milk protein degradation, especially for caseins that are the most abundant and susceptible proteins, is dynamic and may be very sensitive to disease evolution as well as to physiological variables including lactation stage and parity, as highlighted above.

5. Conclusion

This study generated a considerable number on new components to complement the milk peptidome dataset for healthy and mastitic milk available so far [27, 29, 31] and demonstrated that even minimal SCC

increases caused by CNS IMI can alter the milk peptidome, with possible relevant negative consequences on milk quality other than on yield and SCC. Milk peptidome changes were directly related to the SCC and affected mainly caseins, but several peptides were related to CNS-infected milk and others were related to Control milk. Adding to their contribution in understanding the impact of mastitis on milk quality, these might have the potential for mastitis detection, although dedicated efforts for peptide validation with targeted MS methods, as well as for improving robustness and transferability of methods, will be required.

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Author contributions

Study conception and design: MFA, FC, GT, MA, FT, RP, VB. Study coordination: MFA, FC, GT, VB. Animal examination, milk sample collection, milk microbiology: FT, VB. Proteomic analysis and differential proteomics: EMM, GT. Electrophoresis and densitometric analysis: VZ, MFA. Data analysis and interpretation: MFA, EMM. Manuscript drafting: MFA, EMM. 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.

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Figure Legends

Figure 1. Shotgun label-free quantitative peptidomic analysis. **A.** Venn diagram of all the peptides identified in milk samples from healthy (Control) and infected (CNS) cows; **B.** Volcano plot of the differentially abundant peptides determined using Welch's t test (FDR 0.01). Each peptide is represented as a dot and is mapped according to its fold change on the ordinate axis (y), with the p-value by t-test on the abscissa (x). The red and blue dots indicate increased and decreased peptides in CNS, respectively. Grey dots do not meet the FDR criteria.

Figure 2. Milk proteins originating the common and differential peptides identified in the comparison Control vs CNS milk. **A**. The histogram displays the proteins originating the peptides common to Control and CNS milk. Only proteins identified with ≥ 6 peptides are shown. **B**. The histogram displays the proteins originating differential peptides in Control and CNS milk. Only proteins identified with ≥ 1 peptide are shown. Color bars indicate increased (dark grey) or decreased (light grey) peptides in CNS, respectively.

Figure 3. Principal component analysis. A. PCA of the peptidome of all the Control (green) and CNS (red) milk samples. **B.** PCA of milk samples based also on SCC. The plot displays results obtained by grouping and averaging peptidomic data of samples belonging to three different classes: Control (green), CNS-positive with SCC < 300,000 cells/mL (red) and CNS-positive with SCC > 300,000 cells/mL (blue).

Figure 4. Results of the SDS-PAGE densitometric analysis of all milk samples. A. Representative SDS-PAGE profiles of Control (NEG) and CNS-positive milk. Molecular weight references are indicated on the left, while gel areas with the most abundant protein names are indicated on the right as a reference. XD/XO: Xanthine dehydrogenase/oxidase; LTF, lactotransferrin; BSA, bovine serum albumin; MFGE8, lactadherin; IgH, immunoglobulin heavy chain. **B, C, D.** Band volumes and significance of differences obtained when classifying the samples according to bacteriological results and somatic cell thresholds of 100,000 and

200,000 cells/mL, respectively. Error bars indicate standard deviation from the mean. * $P < 0.5$; ** $P < 0.1$, *** $P < 0.001$.

Figure 5. Relative amino acid frequencies at the C-terminus. The figure illustrates the relative distribution of C-terminal amino acids in the peptides INCREASED and DECREASED in the CNS group.

SUPPLEMENTARY FILES

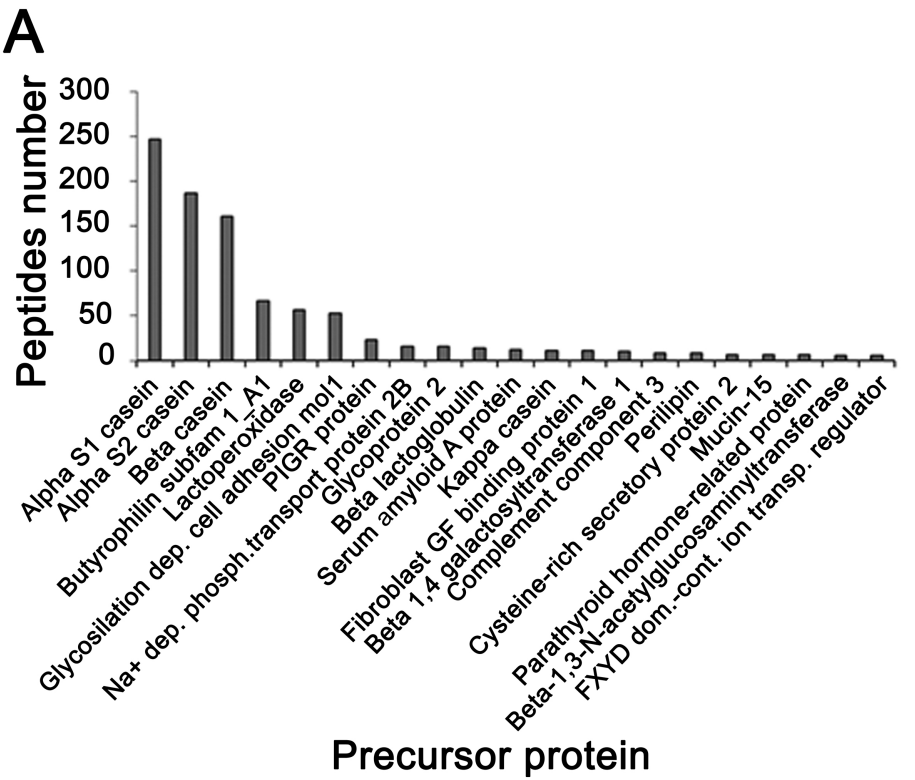
Supplementary File. Table S1. Milk sample characteristics. **Table S2**. List of the peptides INCREASED in the CNS group in the comparison Control *vs* CNS milk. **Table S3.** List of the peptides DECREASED in CNS milk in the comparison Control *vs* CNS milk. **Table S4.** List of the proteins whose proteolysis originate all the peptides detected in Control and CNS samples. **Table S5.** List of the differentially abundant peptides in the comparison Subgroup 2 versus Subgroup 1. The Table reports only the peptides INCREASED in Subgroup 2. **Table S6.** List of the peptides differentially abundant in the comparison Subgroup 2 versus Subgroup 1. The Table reports only the peptides DECREASED in Subgroup2. **Table S7.** List of the peptides differentially abundant in the comparison Subgroup 2 versus Subgroup 3. The endogenous peptides with 100% sequence identity with peptides previously identified in milk during mastitis [29,31,41] are indicated with an "X".

Significance

This is the first investigation on the impact of subclinical CNS mastitis on the bovine milk peptidome. The peptide enrichment strategy combined with a highly sensitive MS/MS analysis enabled the compilation of a very large peptide dataset for healthy and mastitic milk. The comparison of CNS and Control samples, also considering SCC classes, highlighted several peptides with potential for understanding milk protein and peptide dynamics in subclinical mastitis, with possible implications for its detection.


A
CNS
1300
Control
1112
251
1049
63
B
CNS DOWN
89 peptides
CNS UP
112 peptides
-Log Welch value
0
1
2
3
4
5
6
7
8
-6
-4
-2
0
2
4
6
Welch difference

Figure 1


A
Peptides number
300
250
200
150
100
50
0
Alpha S1 casein
Alpha S2 casein
Beta casein
Butyrophilin subfam 1_A1
Lactoperoxidase
Glycosilation dep. cell adhesion mol1
PIGR protein
Na+ dep. phosph.transport protein 2B
Glycoprotein 2
Beta lactoglobulin
Serum amyloid A protein
Kappa casein
Fibroblast GF binding protein 1
Beta 1,4 galactosyltransferase 1
Complement component 3
Perilipin
Cysteine-rich secretory protein 2
Mucin-15
Parathyroid hormone-related protein
Beta-1,3-N-acetylglucosaminyltransferase
FXYD dom.-cont. ion transp. regulator
Precursor protein

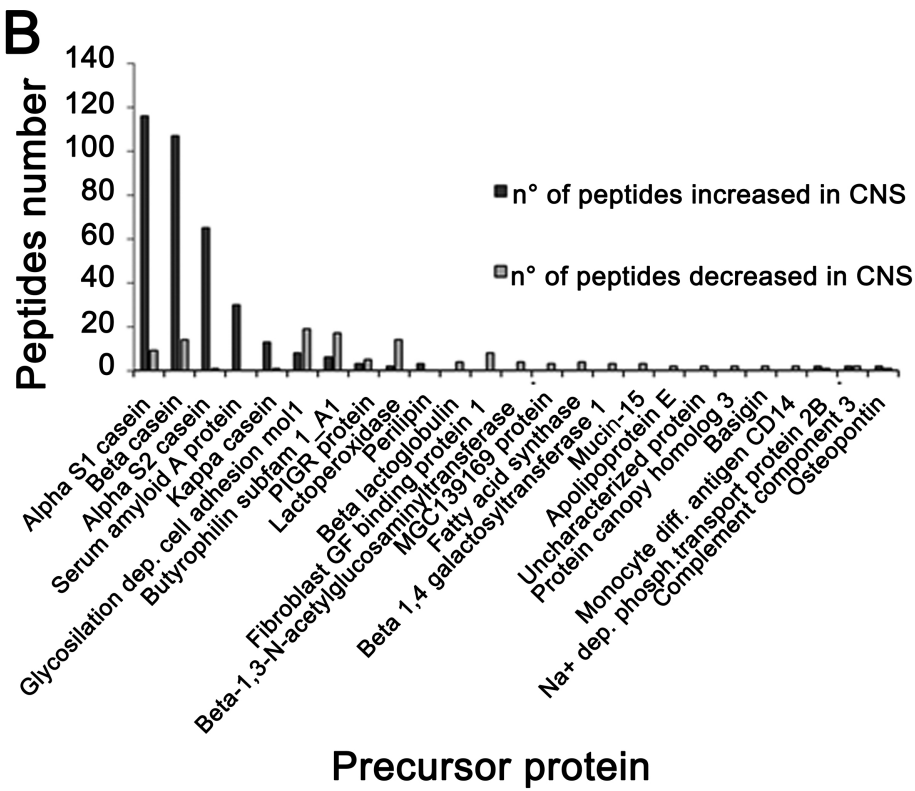

B
Peptides number
140
120
100
80
60
40
20
0
n° of peptides increased in CNS
n° of peptides decreased in CNS
Alpha S1 casein
Beta casein
Alpha S2 casein
Serum amyloid A protein
Kappa casein
Glycosilation dep. cell adhesion mol1
Butyrophilin subfam 1_A1
PIGR protein
Lactoperoxidase
Perilipin
Beta lactoglobulin
Fibroblast GF binding protein 1
Beta-1,3-N-acetylglucosaminyltransferase
MGC139169 protein
Fatty acid synthase
Beta 1,4 galactosyltransferase 1
Mucin-15
Apolipoprotein E
Uncharacterized protein
Protein canopy homolog 3
Basigin
Monocyte diff. antigen CD14
Na+ dep. phosph.transport protein 2B
Complement component 3
Osteopontin
Precursor protein

Figure 2

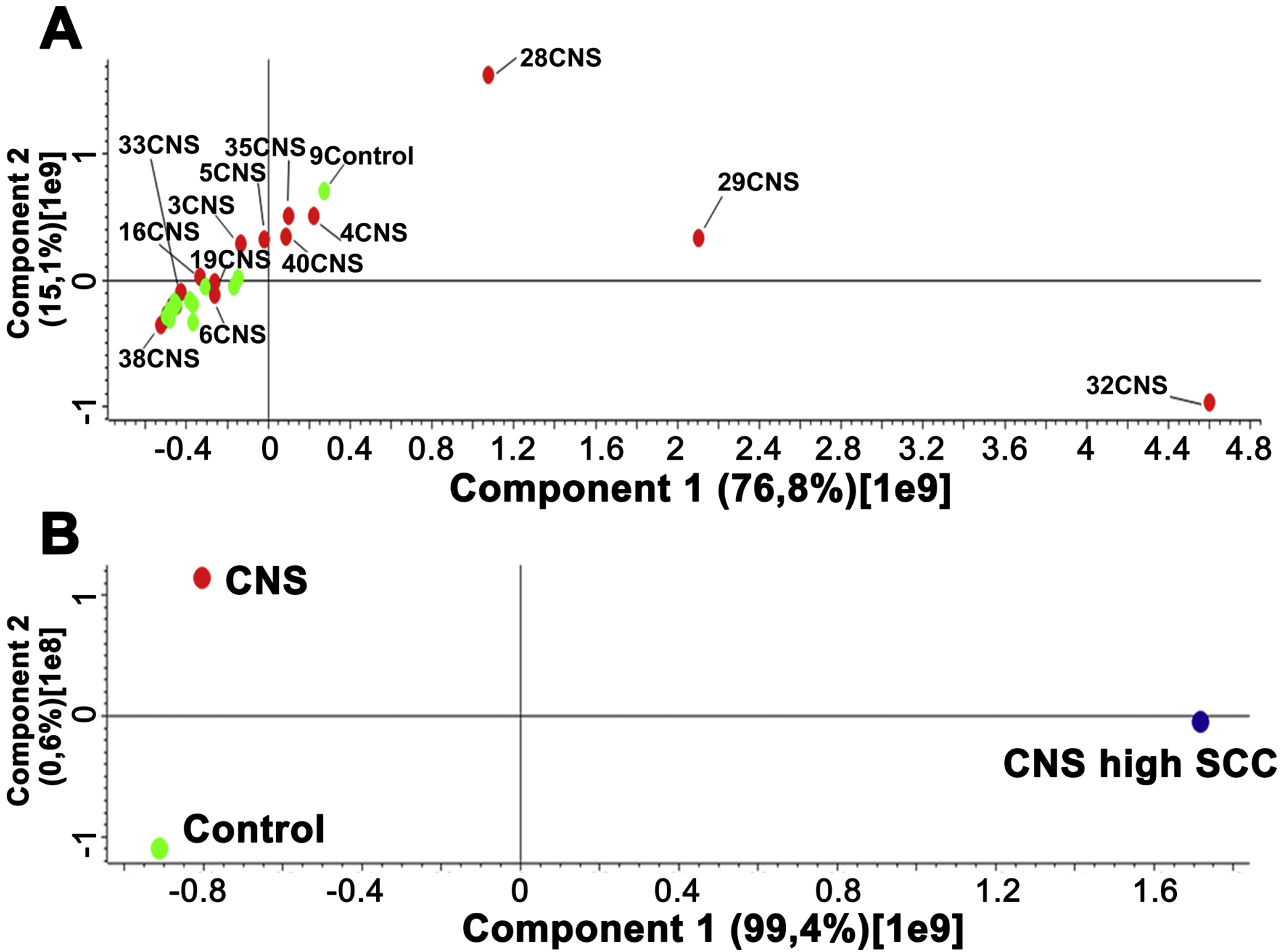

A
28CNS
33CNS
35CNS
9Control
5CNS
29CNS
3CNS
4CNS
16CNS
19CNS
40CNS
6CNS
38CNS
32CNS
Component 2 (15,1%)[1e9]
Component 1 (76,8%)[1e9]
B
CNS
CNS high SCC
Control
Component 2 (0,6%)[1e8]
Component 1 (99,4%)[1e9]

Figure 3

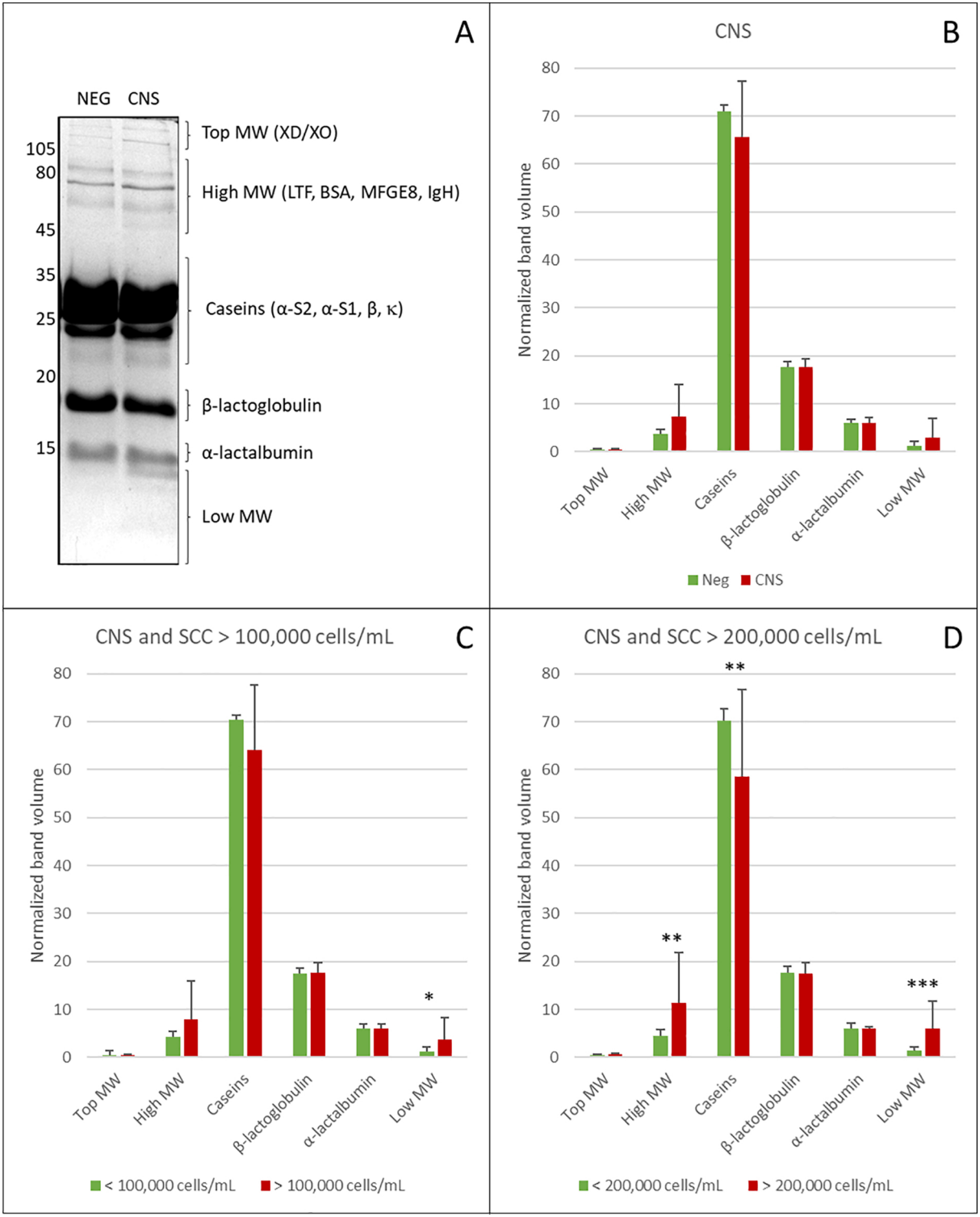

A
NEG
CNS
105
80
45
35
25
20
15
Top MW (XD/XO)
High MW (LTF, BSA, MFGE8, IgH)
Caseins (α-S2, α-S1, β, κ)
β-lactoglobulin
α-lactalbumin
Low MW
B
CNS
Normalized band volume
Top MW
High MW
Caseins
β-lactoglobulin
α-lactalbumin
Low MW
Neg
CNS
C
CNS and SCC > 100,000 cells/mL
Normalized band volume
*
< 100,000 cells/mL
> 100,000 cells/mL
D
CNS and SCC > 200,000 cells/mL
**
**
***
< 200,000 cells/mL
> 200,000 cells/mL

Figure 4

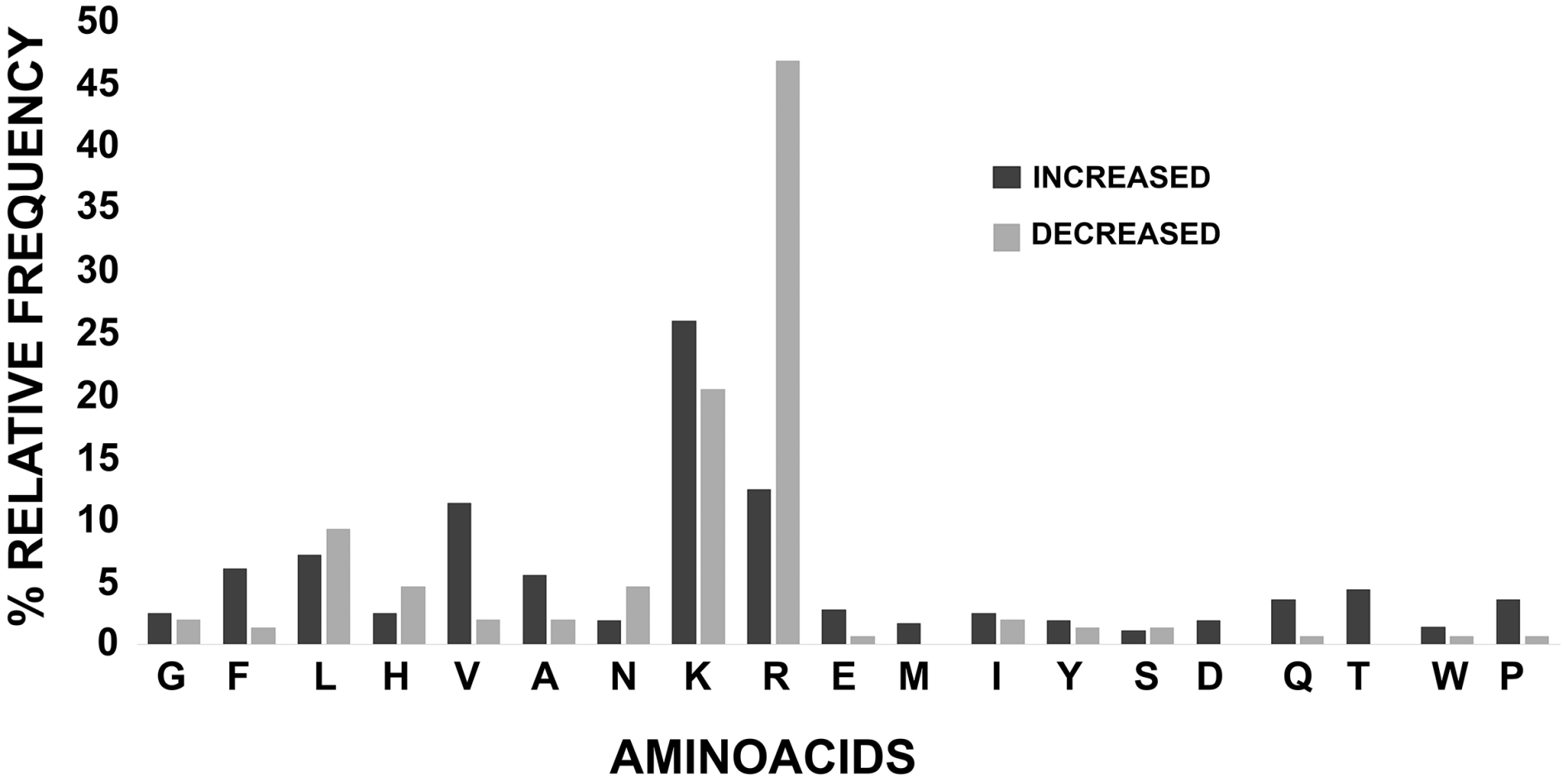

% RELATIVE FREQUENCY
50
45
40
35
30
25
20
15
10
5
0
INCREASED
DECREASED
G F L H V A N K R E M I Y S D Q T W P
AMINOACIDS

Figure 5