A proteomic perspective on the changes in milk proteins due to high somatic cell count

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ABSTRACT

Although cows with subclinical mastitis have no difference in the appearance of their milk, milk composition and milk quality are altered because of the inflammation. To know the changes in milk quality with different somatic cell count (SCC) levels, 5 pooled bovine milk samples with SCC from 10^5 to 10^6 cells/mL were analyzed qualitatively and quantitatively using both one-dimension sodium dodecyl sulfate PAGE and filter-aided sample preparation coupled with dimethyl labeling, both followed by liquid chromatography tandem mass spectrometry. Minor differences were found on the qualitative level in the proteome from milk with different SCC levels, whereas the concentration of milk proteins showed remarkable changes. Not only immune-related proteins (cathelicidins, IGH protein, CD59 molecule, complement regulatory protein, lactadherin), but also proteins with other biological functions (e.g., lipid metabolism: platelet glycoprotein 4, butyrophilin subfamily 1 member A1, perilipin-2) were significantly different in milk from cows with high SCC level compared with low SCC level. The increased concentration of protease inhibitors in the milk with higher SCC levels may suggest a protective role in the mammary gland against protease activity. Prostaglandin-H2 D-isomerase showed a linear relation with SCC, which was confirmed with an ELISA. However, the correlation coefficient was lower in individual cows compared with bulk milk. These results indicate that prostaglandin-H2 D-isomerase may be used as an indicator to evaluate bulk milk quality and thereby reduce the economic loss in the dairy industry. The results from this study reflect the biological phenomena occurring during subclinical mastitis and in addition provide a potential indicator for the detection of bulk milk with high SCC.

Key words: proteomics, mastitis, mammary gland, somatic cell count

INTRODUCTION

Mastitis, an inflammation of the mammary gland, is one of the most devastating diseases affecting dairy cows, which results in changes of milk appearance, milk composition, and SCC (Forsbäck et al., 2010; Awale et al., 2012). A decrease in CN and whey protein concentrations has been reported to occur during mastitis (Hogarth et al., 2004). The lactose, sodium, and potassium contents were also found to be changed in concentration in milk from cows with mastitis due to leaky tight junctions (Hagiwara et al., 2003; Lindmark-Månsson et al., 2006). Inflammation of the mammary gland during mastitis easily develops in response to infection and can lead to severe damage to the milk secretory tissue of the udder, resulting in a reduction in milk production and deteriorated milk quality (Hogarth et al., 2004). Mastitis is therefore considered as a major source of economic losses on dairy farms and a serious burden on the dairy producers.

Although milk from cows with subclinical mastitis does not have visible changes in the appearance, as mentioned above, changes in milk composition and high SCC do occur. Milk from quarters with subclinical mastitis showed elevated levels of sodium, chloride, albumin, lactate dehydrogenase activity, and immunoglobulins as well as reduced levels of α -LA, β -LG, calcium, inorganic phosphorus, and potassium (Batavani et al., 2007). It also contributes to an average milk production loss of 470 kg per primiparous dairy cow and 740 kg per multiparous dairy cow during the full lactation with each unit increase in $\text{Log}_{10}(\text{SCC})$ (Kolde-weijs et al., 1999). Thus, the greater the SCC increase, the greater the production loss. In addition, subclinical mastitis causes a similar reduction of reproductive performance as mastitis (Schrack et al., 2001). High SCC in milk has been considered as the only evidence that helps in the diagnosis of subclinical mastitis (Turk et al., 2012). Therefore, identifying new biomarkers for subclinical mastitis may help to develop an easy test aiming at predicting milk suitability for further milk processing. Consequently, it is interesting to study the differences of the milk proteome between healthy

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cows and cows with high SCC for understanding the variations of proteins expressed in milk from cows with subclinical mastitis. This may contribute to potential biomarker discovery for the detection of bovine subclinical mastitis.

Compared with the effect of subclinical mastitis, the effect of clinical mastitis has been studied much more widely. For instance, Yang et al. (2009) detected differently expressed proteins in the mammary gland from mastitis cows and healthy cows, such as hemoglobin, β -CN, κ -CN, and tryptophanyl-tRNA-synthetase, which all showed lower concentrations in milk from mastitis cows, whereas cytochrome C oxidase and annexin V showed higher abundance in milk from mastitis cows (Yang et al., 2009). Proteases, including plasmin, cathepsin B and D, elastase, and amino- and carboxypeptidases, were identified in bovine milk following infusion with lipoteichoic acid isolated from *Staphylococcus aureus* (Larsen et al., 2010). Low-abundant inflammation markers such as serotransferrin, fibrinogen β chain, S100 calcium-binding protein A12, and the antimicrobial cathelicidins were shown to be present in relative high amounts in milk 12 h after infusion with *Escherichia coli* lipopolysaccharide (Hinz et al., 2012). Only few studies have been carried out on the milk proteome of cows with subclinical mastitis. Safi et al. (2009) found that acute phase proteins (haptoglobin and amyloid A) increased in milk from cows with subclinical mastitis (Safi et al., 2009). Serpin A3-1, vitronectin-like protein, and complement factor H were shown to be upregulated in milk from subclinical-mastitis cows in comparison with healthy cows (Turk et al., 2012).

The objective of the present study was to investigate the influence of high SCC (up to 10^6 cells/mL) on the milk proteins in bovine milk by one-dimensional SDS-PAGE and also by filter-aided sample preparation (FASP) combined with dimethyl labeling, both followed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Using shotgun proteomics techniques to determine variation in protein levels in milk from infected cows will increase our understanding of the influence of high SCC levels on the protein composition of milk, thereby providing potential biomarkers for detecting milk with a high SCC level.

MATERIALS AND METHODS

Sample Collection

A total of 100 cows were used in this study. Milk samples were a mixture from all 4 quarters of each cow. Five groups were made, consisting of pooled samples from 20 cows with similar SCC, which are SCC1 ($<10^5$

cells/mL); SCC2 ($2.25\text{--}2.75 \times 10^5$ cells/mL); SCC3 ($4.8\text{--}5.3 \times 10^5$ cells/mL); SCC4 ($7\text{--}8 \times 10^5$ cells/mL); and SCC5 ($9.25\text{--}10.75 \times 10^5$ cells/mL). The sample with lowest SCC (SCC1) will be referred to as "low SCC." All other samples (SCC2–SCC5) represent samples with increased cell counts and will be referred to as "high SCC," because SCC 2.5×10^5 cells/mL (SCC2) is considered the threshold for subclinical mastitis (Turk et al., 2012).

Sodium azide (0.02% wt/wt) and bronopol (0.0005% wt/wt) were added to prevent bacterial growth in these pooled milk samples supplied by Qlip (Dutch milk controlling station, Zutphen, the Netherlands). Several (28) individual samples with SCC ranging from 1×10^5 to 8.5×10^5 cells/mL were also collected from Qlip for determining the relation between prostaglandin-H2 D-isomerase (PTGDS) and SCC in the milk serum of individual cows.

Milk-Composition Analysis and Proteomics Technique

Milk samples were analyzed for SCC, DM, protein, fat, and lactose contents by CombiFoss 5000 by Qlip. The proteomics methods used in this study are based on previous articles (Wisniewski et al., 2009; Hettinga et al., 2011; Lu et al., 2011).

Milk Serum Separation. To separate milk serum, pooled samples were centrifuged at $1,500 \times g$ for 10 min at 10°C (Beckman Coulter AvantiJ-26 XP centrifuge, rotor JA-25.15, Brea, CA). The pellet was removed, and the obtained supernatant was transferred to the ultracentrifuge tubes followed by ultracentrifugation at $100,000 \times g$ for 90 min at 30°C (Beckman L-60, rotor 70 Ti, Beckman Coulter). After ultracentrifugation, samples were separated into 3 phases. Milk serum, in the middle layer, was separated and used for the proteomics sample preparation, as described below.

SDS-PAGE. Sodium dodecyl sulfate-PAGE was used to further separate milk proteins. Samples were subjected to one-dimensional SDS-PAGE using precast 12% Precise Protein Gels with HEPES buffer (Thermo Fisher Scientific Inc., Waltham, MA). The thawed protein samples were mixed 1:1 with $2\times$ sample buffer (125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.01% bromophenol blue in water); just before use, 5% β -mercaptoethanol was added, and the samples were heated for 5 min at 95°C . Gels were loaded with approximately 30 μg of protein per well. The gels were run for 45 min at 130 V and then fixed and stained with the Colloidal Blue Staining Kit (LC6025, Invitrogen, Carlsbad, CA) for 4 h and finally destained overnight in water.

In-Gel Digestion. Except when stated otherwise, all NH_4HCO_3 (ABC, 0.05 M) buffers used in this section were pH 8. After each step, the samples were sonicated for 1 min followed by centrifugation. For each sample, the gel lane was cut into 8 slices. Each slice was cut into pieces of maximum 1-mm³ size using a scalpel and transferred to a low-binding microcentrifuge tube (0030 108.094, Eppendorf, Hamburg, Germany). The gel pieces were washed twice with water. The proteins were reduced by incubation in 0.05 M dithiothreitol for 1 h at 60°C followed by incubation in 0.1 M iodoacetamide for 1 h at room temperature in the dark. After carboxamidomethylation, the gel pieces were washed 3 times with 0.05 M ABC buffer. The gel pieces were then frozen and thawed 3 times to increase the accessibility for trypsin. Next, the samples were rehydrated in 20 μL of freshly prepared trypsin solution (10 ng/ μL). Extra 0.05 M ABC buffer was added to completely cover the gel pieces before they were incubated overnight at room temperature. The next day, the basic supernatant of digest solution was transferred to a clean low-binding microcentrifuge tube. The gel pieces were extracted twice with 10 μL of 5% trifluoroacetic acid in water and 10 μL of 10% acetonitrile/1% trifluoroacetic acid, respectively, with sonication after each extraction step. The supernatants after every sonication were added to the same low-binding microcentrifuge tube. Final pH of the peptide mixture was checked using pH paper.

FASP. Filter-aided sample preparation was used to digest milk proteins into peptides for identification and quantification. Milk serum samples (20 μL) were diluted in 0.1 M Tris/HCl pH 8.0 + 4% SDS + 0.1 M dithiothreitol to get a 1 $\mu\text{g}/\mu\text{L}$ protein solution. Samples were then incubated for 5 min at 95°C and then cooled down to room temperature, and 10 μL of sample was directly added to a Pall 3 K omega filter (10–20 kDa cut off, OD003C34; Pall, Washington, NY) and centrifuged at $15,871 \times g$ for 1 min. A total of 100 μL of 8 M urea in 0.1 M Tris/HCl pH 8.0 (UT) was added to the filter and centrifuged at $15,871 \times g$ for 30 min. A total of 100 μL of 0.05 M iodoacetamide in UT was added and incubated for 10 min at room temperature and then centrifuged at $15,871 \times g$ for 30 min to alkylate cysteine residues within proteins. Another 3 centrifugations at $15,871 \times g$ for 30 min were carried out after adding 110, 120, and 130 μL of UT, respectively, to remove remaining detergent. After that, 140 μL of ABC was added to the filter unit and centrifuged at $15,871 \times g$ for 30 min to provide an optimal pH for the enzymatic cleavage. Then, the filter was transferred to a new low-binding Eppendorf tube. A total of 100 μL of ABC containing 0.5 μg of trypsin was added and centrifuged at $15,871 \times g$ for 30 min to collect peptides after 2 h of incubation at 45°C. Finally, the filter was

removed, and 3.5 μL of 10% trifluoroacetic acid was added to adjust the pH value of the sample to around 2. These samples were ready for dimethyl labeling.

Dimethyl Labeling. To determine the precise changes in proteins from milk with low SCC and high SCC, dimethyl labeling was used. Milk sample with low SCC (SCC1) was labeled with the light reagent (the mix of HCHO and sodium cyanoborohydride) as reference, whereas milk samples with high SCC (SCC2, SCC3, SCC4, SCC5) were labeled with the heavy reagent [the mix of formaldehyde (DCDO) and sodium cyanoborohydride]. The dimethyl labeling method was carried out using on-column dimethyl labeling according to (Boersema et al., 2009). Stage tips containing 2 mg of LiChroprep C18 (25- μm particles; Merck Millipore, Amsterdam, the Netherlands) column material (C18+ Stage tip) columns were made in house. The C18+ Stage tip column was washed 3 times with 200 μL of methanol. The column was conditioned with 100 μL of 1 mL/L formic acid in water (HCOOH), and then samples were loaded on the C18+ Stage tip column. The column was washed with 100 μL of 1 mL/L HCOOH and then slowly flushed with 100 μL of labeling reagent (0.2% CH_2O or CD_2O and 30 mM cyanoborohydride in 50 mM phosphate buffer pH 7.5) in about 10 min. The column was washed again with 200 μL of 1 mL/L HCOOH. Finally, the labeled peptides were eluted with 50 μL of 70% acetonitrile/30% 1 mL/L HCOOH from the C18+ Stage tip columns. The samples were then dried in a Speed-Vac (AG 22331, Eppendorf, Hamburg, Germany) at 35°C for 1.5 to 2 h until the volume of each sample decreased to 15 μL or less. The pairs of light dimethyl label and heavy dimethyl label were then mixed up to 100 μL by adding 1 mL/L HCOOH. These samples were ready for analysis by LC-MS/MS.

LC-MS/MS. A total of 18 μL of the trypsin-digested milk fractions was injected on a 0.10×30 mm Magic C18AQ 200A 5 μm beads (Michrom Bioresources Inc., Auburn, CA) preconcentration column (prepared in house) at a maximum pressure of 27,000 kPa. Peptides were eluted from the preconcentration column onto a 0.10×200 mm Prontosil 300-3-C18H analytical column (Prontosil, Bischoff, Germany) with an acetonitrile gradient at a flow of 0.5 $\mu\text{L}/\text{min}$, using gradient elution from 9 to 34% acetonitrile in water with 0.5 vol/vol % acetic acid in 50 min. The column was washed using an increase in the percentage acetonitrile to 80% (with 20% water and 0.5 vol/vol % acetic acid in the acetonitrile and the water) in 3 min. Between the preconcentration and analytical columns, an electrospray potential of 3.5 kV was applied directly to the eluent via a solid 0.5-mm platinum electrode fitted into a P777 Upchurch microcross (IDEX, Oak Harbor, WA). Full scan positive mode FTMS spectra

were measured between m/z 380 and 1,400 on an LTQ-Orbitrap XL (Thermo Electron, San Jose, CA). The CID fragmented MS/MS scans of the 4 most abundant doubly and triply charged peaks in the FTMS scan were recorded in data-dependent mode in the linear trap (MS/MS threshold = 5.000).

Data Analysis. Each run with all MS/MS spectra obtained was analyzed with Maxquant1.3.0.5 with Andromeda search engine (Cox and Mann, 2008; Cox et al., 2011). Carboxamidomethylation of cysteines was set as a fixed modification (enzyme = trypsin, maximally 2 missed cleavages, peptide tolerance 20 ppm, fragment ions tolerance 0.5 amu). Oxidation of methionine, N-terminal acetylation and deamidation of asparagine or glutamine were set as variable modifications for both identification and quantification. The bovine reference database for peptide and protein matching was downloaded from Uniprot (<http://www.uniprot.org/>) with reverse sequences generated by Maxquant. A set of 31 protein sequences of common contaminants was added including trypsin (P00760, bovine), trypsin (P00761, porcine), keratin K22E (P35908, human), keratin K1C9 (P35527, human), keratin K2C1 (P04264, human), and keratin K1C1 (P35527, human). A maximum of 2 missed cleavages were allowed and mass deviation of 0.5 Da was set as a limitation for MS/MS peaks and maximally 6 ppm deviation on the peptide m/z during the main search. The false discovery rate was set to 1% on both peptide and protein levels. The length of peptides was set to be at least 6 amino acids. Finally, proteins were displayed based on minimally 2 distinct peptides of which at least one was unique.

Dimethyl labeling was based on doublets with dimethLys0 and dimethNter0 as light (L) and dimethLys4 and dimethNter4 as heavy (H). Razor peptides (peptides that are nonunique, which are assigned to the protein with the most other peptides) and unique peptides (peptides that are unique to one protein) were used for quantification. Normalized heavy:light (H/L) ratios calculated as the median of all dimethyl-labeled peptide-pair ratios that belong to one protein were used for further statistical analysis. In each liquid chromatography-mass spectrometry run, peptide ratios were normalized, so that the median of their logarithms was zero, which corrects for unequal protein loading (Cox and Mann, 2008; Cox et al., 2011). Also, the intensity-based absolute quantification algorithm was used, which calculates absolute protein amounts as the sum of all peptide intensities divided by the number of theoretically observable tryptic peptides. The intensity-based absolute quantification value has been reported to have a good correlation with known absolute protein amounts over at least 4 orders of magnitude (Malmström et al., 2009; Schwanhauser et al., 2011).

The function of the identified proteins was checked in the UniProtKB database released April 2012 (<http://www.uniprot.org/>). Gene Ontology enrichment analysis was done using DAVID bioinformatics Resources 6.7 (Dennis et al., 2003; <http://david.abcc.ncifcrf.gov/>). The significant analysis in the changes of milk composition was conducted by one-sample *t*-test in SPSS (Version 21, IBM Corp., Armonk, NY), which has been used in the significant analysis of microarray-based gene expression (Patterson et al., 2006). For the changes of individual proteins, a 2-fold change was used as the significant cut-off in this study, which has been reported in the previous milk proteomics studies done by FASP and dimethyl labeling (Lu et al., 2011, 2013). The linear correlation analysis between SCC and the relative concentration of milk proteins was performed in SPSS (Version 21, IBM Corp.).

ELISA

An ELISA was used to confirm the correlation between PTGDS and SCC that was found in the LC-MS/MS analysis. The samples were diluted 5,000-fold aiming at the final concentration of PTGDS ranging from 93.75 to 6,000 pg/mL. The concentration of PTGDS in the milk serum was detected in duplicates by the ELISA kit (bovine PTGDS, MBS942806) bought from EMELCA Bioscience Company (Breda, the Netherlands). The ELISA test, including creation of a standard curve, was performed according to the manufacturer's instructions. A 4-parameter logistic model, $y = [d + (a - d)]/[1 + (x/c)^b]$, was used for calibration by MasterPlex ReaderFit (Hitachi Solutions America Ltd., San Bruno, CA). The standard curve $y = 2.69 - 2.65/(1 + x/673.33)^{-1.41}$ ($r^2 = 0.988$) was used to calculate the concentration of PTGDS in the samples based on the response value from the micro plate reader.

RESULTS

Identification of the Milk Serum Proteome

Table 1 shows the composition of the milk samples of the 5 groups (20 cows per group) with different SCC levels. No significant differences were found in milk composition among these 5 groups ($P > 0.05$). After proteomic analysis using one-dimensional SDS-PAGE combined with nanoLC-Orbitrap-MS/MS (Thermo Electron, San Jose, CA), a total of 273 proteins from the 5 pooled samples were identified (Supplemental Table S1; <http://dx.doi.org/10.3168/jds.2014-9279>). The number of identified proteins had minor differences (less than 10%) between the groups. From the group with the lowest SCC to the group with the highest

Figure 1. Functional ontology classification of proteins in bovine milk with different level of SCC (SCC1 = $<10^5$ cells/mL; SCC2 = 2.5×10^5 cells/mL; SCC3 = 4×10^5 cells/mL; SCC4 = 7.5×10^5 cells/mL; SCC5 = 10^6 cells/mL). Cell includes cell adhesion, cell mobility, cell growth, and cell apoptosis.

SCC, 217, 187, 201, 208, and 217 proteins were identified, respectively. The proteins identified were grouped based on their biological functions and subcellular locations according to Gene Ontology using DAVID (Dennis et al., 2003) as shown in Figure 1 and 2. Most of the proteins identified were linked to immunity, transport, cell functions (including cell adhesion, cell mobility, cell growth, and cell apoptosis), or enzymes. Based on the subcellular location, the 3 major groups, secreted, membrane, and cytoplasm, constituted more than 90% of the total proteins. Secreted was the dominant subcellular location and accounted for around 45% of the total proteins. Even though proteins originating from endoplasmic reticulum, Golgi, lysosome, and nucleus accounted for a very little part of identified proteins, they were present in all of these samples as shown in Figure 2. The number of identified proteins based on

their biological functions and subcellular locations did not change with the increase of SCC.

Quantification of the Milk Serum Proteome

To know the influence of high SCC on the expression level of the milk proteome, FASP combined with dimethyl labeling, followed by nanoLC-Orbitrap-MS/MS, was used for quantitative analysis. A total of 100 proteins were quantified (as shown in Supplemental Table S2; <http://dx.doi.org/10.3168/jds.2014-9279>). The biological functions and relative concentration of the 56 common proteins are shown in Table 2. A specific group of immune-related proteins, including all cathelicidins, PTGDS, Ig gamma-2 chain C region (**IGHG2**), and inter- α -trypsin inhibitor heavy chain H4 (**ITIH4**),

Table 1. Composition of the milk samples with different level of SCC (SCC1 = $<10^5$ cells/mL; SCC2 = 2.5×10^5 cells/mL; SCC3 = 4×10^5 cells/mL; SCC4 = 7.5×10^5 cells/mL; SCC5 = 10^6 cells/mL)

Milk composition	SCC1	SCC2	SCC3	SCC4	SCC5	One-sample test (test value = 0)	
						Significance (2-tailed)	Mean difference
DM (%)	13.8	13.46	13.24	13.42	13.22	0.943	0.008
Protein (%)	3.72	3.65	3.91	3.68	3.54	1	0.000
Fat (%)	4.42	4.48	4.24	4.52	4.46	1	0.000
Lactose (%)	4.84	4.49	4.16	4.34	4.37	0.973	0.004

Figure 2. Subcellular location classification of proteins in bovine milk with different level of SCC (SCC1 = $<10^5$ cells/mL; SCC2 = 2.5×10^5 cells/mL; SCC3 = 4×10^5 cells/mL; SCC4 = 7.5×10^5 cells/mL; SCC5 = 10^6 cells/mL). ER = endoplasmic reticulum.

showed an increase in the milk samples with high SCC compared with low SCC.

Next, a comparison was made between milk from cows with highest SCC (SCC5, 10^6 cells/mL) and milk with lowest SCC (SCC1, $<10^5$ cells/mL), as shown in Figure 3. These significantly different proteins had more than 2-fold changes ($\log_2$ ratio beyond -1 and 1), which was reported as the cut-off level by previous studies (Lu et al., 2011, 2013). Nine of the quantified proteins showed a more than 2-fold increase in milk with highest SCC, as shown in Figure 3, whereas 12 of them showed a more than 2-fold decrease. Among these upregulated proteins, most are related to the immune system, such as cathelicidins, α -1-microglobulin (AMBP), IGHG2, ITIH4, and S100 calcium-binding protein A8 (S100A8). The most significantly changed protein [cathelicidin 4 (CATHL4)] showed an almost 10-fold increase in the milk from cows with the highest SCC. The upregulation of these proteins was similar to what we found in a preliminary experiment conducted by the same techniques (Supplemental Table S3; <http://dx.doi.org/10.3168/jds.2014-9279>). Of the downregulated proteins, platelet glycoprotein 4 (CD36), butyrophilin subfamily 1 member A1 (BTN1A1), and perilipin-2 (PLIN2) are related to lipid synthesis and secretion; ATP-binding cassette subfamily G member 2 (ABCG2) is related to milk secretion; lactadherin (MFGE8) is involved in cell apoptosis; and CD59 glycoprotein (CD59) is related to the immune system. The concentration of platelet glycoprotein 4 was about 20-fold lower in milk from cows with highest SCC, as shown in Figure 3.

In addition, the correlation between the concentration of proteins and SCC was calculated to find potential biomarkers for subclinical mastitis (Supplemental Table S4; <http://dx.doi.org/10.3168/jds.2014-9279>). Among the immune-related proteins, the concentration of PTGDS showed the strongest linear relation with SCC (Figure 4A). The increase in both PTGDS and SCC had the same order of magnitude, showing a 10-fold increase in PTGDS with a 10-fold increase in SCC. A similar correlation between PTGDS and SCC was also found in a preliminary experiment as shown in Supplemental Table S3 (<http://dx.doi.org/10.3168/jds.2014-9279>). Next, this correlation was confirmed by an ELISA (Figure 4B). Finally, to know whether the concentration of PTGDS correlates with SCC in individual cows, the concentration of PTGDS from 28 individual milk samples with SCC ranging from 1×10^5 to 8.5×10^5 cells/mL were determined using the same ELISA assay, as shown in Figure 4C. Even though the correlation between PTGDS and SCC in the individual cows was not very strong, there was a similar direction in the relation, as well as similar absolute values, in the individual cows.

DISCUSSION

Protein Identification

In the current study, a total of 273 proteins were identified in bovine milk serum by one-dimensional SDS-PAGE followed by nanoLC-Orbitrap-MS/MS.

A similar number of proteins, 269 (including 232 in bovine milk fat globule membrane and 192 in bovine milk serum), were identified in a previous study using the same proteomics techniques (Hettinga et al., 2011). We found several low-abundant immune-related proteins [S100 calcium-binding protein A2 (S100A2), S100 calcium-binding protein A8 (S100A8), S100 calcium-binding protein A9 (S100A9), serum amyloid A-1 protein (SAA1), serum amyloid A-3 protein (SAA3), cathelicidins] in milk from both healthy cows and cows with high SCC (up to 10^6 cells/mL), whereas these proteins were previously only identified in bovine milk from cows with clinical mastitis (Alonso-Fauste et al., 2012). This may be attributed to the high sensitivity of the proteomics technique applied in this study.

The absence of changes in protein and DM contents (Table 1) in milk with high SCC is in accordance with an earlier study (Ogola et al., 2007). The decreasing trend in lactose (Table 1) agrees with what has been reported previously (Ogola et al., 2007). Moreover, the number of identified proteins and their distribution over biological functions and subcellular locations was similar in milk samples at different SCC levels (Figures 1 and 2). Milk at all SCC levels contained a large number of immune-related proteins as well as secreted proteins (Figures 1 and 2). The data show that the difference between milk from healthy cows and cows with subclinical mastitis is not related to the presence or absence of proteins but is caused by their expression levels, as will be discussed next.

Protein Quantification

To study differences in expression levels of milk proteins, FASP combined with dimethyl labeling was used for quantitative analysis. The results of this quantitative analysis show many differences in the concentration of proteins, including both immune-related proteins and proteins with other biological functions, such as cellular functions, transport, enzymes, and protease inhibitors, as shown in Supplemental Table S2 (<http://dx.doi.org/10.3168/jds.2014-9279>). The increase of free CN (or CN peptides) in the milk serum with high SCC samples (Table 2) is consistent with the decrease of micellar CN described in a previous study (Forsbäck et al., 2010). As SCC increased, proteolysis also increased (Larsen et al., 2010), which thereby results in the increase of free CN, or CN peptides, in the milk serum. A more comprehensive study of these CN peptides would, however, require a different technological approach than what was used in this study. The concentration of specific immune-related proteins (all cathelicidins, IGHG2, ITIH4) increased in all samples with high SCC but not proportionally with SCC (Table 2). Chiaradia,

et al. (2013) reported that also in ovine milk, the proteins upregulated with mammary gland infection did not increase proportionately with SCC (Chiaradia et al., 2013). They believed this could be due to different processes occurring during subclinical mastitis because the increase of SCC in milk is not only caused by the bacterial infection but also by the stressful conditions and a probable metabolic impairment of the mammary gland (Chiaradia et al., 2013).

Upregulated Proteins in Milk from Cows with High SCC

There were 10 proteins that showed a more than 2-fold increase in milk serum with an average SCC of 10^6 cells/mL compared with milk serum with an average SCC of 10^5 cells/mL, as shown in Figure 3. Of these upregulated proteins, 3 proteins were cathelicidins. Their concentration increased significantly in milk serum from cows with subclinical mastitis (Figure 3), which was also found in milk from cows with mastitis after *E. coli* or *Streptococcus uberis* invasion (Lippolis and Reinhardt, 2005; Boehmer et al., 2008; Smolenski et al., 2011). This may be related to the antimicrobial activity of cathelicidins as well as their role in modulation of the innate and adaptive immune response (Teclé et al., 2010). The significant increase in immunoglobulins (IGHG2 and IGHK) and acute phase proteins (S100 calcium-binding protein A8 and ITIH4) in the milk with high SCC (Figure 3) has been discussed frequently in the previous studies (Caffin and Poutrel, 1988; Mitterhuemer et al., 2010; Alonso-Fauste et al., 2012; Huang et al., 2014). Protease inhibitors play a crucial role in the regulation of protease activity originating from neutrophils (Owen and Campbell, 1999). Serine protease inhibitors have been shown to protect tissue against damage that can be caused by proteases secreted by neutrophils during an infection (Thompson and Ohlsson, 1986). For this reason, the observed increase in SERPINA3 in the milk with high SCC (Figure 3) might be related to the protection of mammary gland cells.

Downregulated Proteins in Milk from Cows with High SCC

As shown in Figure 3, 12 proteins were more than 2-fold downregulated in milk with the highest versus the lowest SCC. Most of the downregulated proteins were related to other biological functions than immune protection, for example, lipid metabolism (platelet glycoprotein 4 20-fold, butyrophilin subfamily 1 member A1 4-fold, and perilipin-2 6-fold). The downregulation of all these proteins has been reported before in milk

Table 2. The ratios of common proteins and immune-related proteins between samples at different SCC levels (SCC1 = <10⁵ cells/mL; SCC2 = 2.5 × 10⁵ cells/mL; SCC3 = 4 × 10⁵ cells/mL; SCC4 = 7.5 × 10⁵ cells/mL; SCC5 = 10⁶ cells/mL)

Uniprot ID	Protein name	Gene name	Biological function ¹	SCC2/ SCC1 ²	SCC3/ SCC1 ²	SCC4/ SCC1 ²	SCC5/ SCC1 ²
P33046	Cathelicidin-4	<i>CATHL4</i>	Immunity	3.64	NQ	NQ	9.82
O02853	Prostaglandin-H2 D-isomerase	<i>PTGDS</i>	Enzyme	1.98	3.163	5.849	7.144
P22226	Cathelicidin-1	<i>CATHL1</i>	Immunity	3.302	7.441	6.148	7.012
P19660	Cathelicidin-2	<i>CATHL2</i>	Immunity	NQ	4.96	4.75	4.2
G3N0V0	Ig γ -2 chain C region	<i>IGHG2</i>	Immunity	2.119	2.755	1.77	2.638
Q3T052	Inter- α -trypsin inhibitor heavy chain H4	<i>ITIH4</i>	Immunity	NQ	3.12	1.36	2.19
P02754	β -LG	<i>LGB</i>	Transport	0.601	0.456	4.033	2.092
G3N1U4	Serpin A3-8	<i>SERPINA3-8</i>	Protease inhibitor	1.18	1.446	1.152	2.042
P61823	Ribonuclease pancreatic	<i>RNASE1</i>	Enzyme	1.309	1.069	2.429	1.929
P17697	Clusterin	<i>CLU</i>	Immunity	1.764	5.09	3.621	1.733
P60712	Actin, cytoplasmic 1	<i>ACTB</i>	Protein synthesis	1.756	1.198	2.224	1.63
Q0IIA2	Odorant-binding protein-like	<i>MGC151921</i>	Transport	1.597	3.067	1.353	1.507
P24627	Lactotransferrin	<i>LTF</i>	Immunity	2.082	2.735	2.717	1.494
A7YWR0	Apolipoprotein E	<i>APOE</i>	Transport	1.006	1.577	2.019	1.473
B0JYP6	IGK protein	<i>IGK</i>	Immunity	1.289	2.021	1.864	1.351
P79345	Epididymal secretory protein E1	<i>NPC2</i>	Transport	1.737	1.016	1.332	1.348
P00735	Prothrombin	<i>F2</i>	Immunity	NQ	0.95	NQ	1.34
G3N1R1	Hyaluronan and proteoglycan link protein 1	<i>HAPLN1</i>	Immunity	1.193	2.557	2.248	1.328
P80195	Glycosylation-dependent cell adhesion molecule 1	<i>GLYCAM1</i>	Immunity	0.83	1.021	1.599	1.326
Q29443	Serotransferrin	<i>TF</i>	Transport	1.344	1.549	1.752	1.308
Q2UVX4	Complement C3	<i>C3</i>	Immunity	1.565	1.502	1.589	1.283
P80025	Lactoperoxidase	<i>LPO</i>	Immunity	1.078	0.865	1.271	1.279
P31096	Osteopontin	<i>SPP1</i>	Cell	1.603	2.982	0.948	1.276
P02663	α -S2-CN	<i>CSN1S2</i>	Transport	0.982	1.163	2.193	1.259
A5D7Q2	TAP binding protein (Tapasin)	<i>TAPBP</i>	Immunity	2.064	1.53	2.339	1.211
Q3ZCH5	Zinc- α -2-glycoprotein	<i>AZGP1</i>	Immunity	1.138	1.045	0.986	1.195
P81265	Polymeric immunoglobulin receptor	<i>PIGR</i>	Immunity	1.212	1.09	1.327	1.167
Q3T101	IGL@	<i>IGL@</i>	Immunity	1.606	2.202	1.887	1.151
Q2KJF1	α -1B-glycoprotein	<i>A1BG</i>	Immunity	0.843	1.08	1.644	1.111
P02666	β -CN	<i>CSN2</i>	Transport	0.423	1.516	3.407	1.084
P02662	α -S1-CN	<i>CSN1S1</i>	Transport	0.458	0.88	2.284	1.062
P01044	Kininogen-1	<i>KNG1</i>	Coagulation	1.057	1.222	1.113	1.053
P08037	β -1,4-galactosyltransferase 1	<i>B4GALT1</i>	Immunity	1.258	1.148	2.382	1.039
Q0IIH5	Nucleobindin 2	<i>NUCB2</i>	Transport	1.08	0.885	0.774	1.033
P34955	α -1-antiproteinase	<i>SERPINA1</i>	Protease inhibitor	1.773	1.105	0.982	1.01
P41361	Antithrombin-III	<i>SERPINC1</i>	Coagulation	1.131	0.915	0.98	0.991
B5B0D4	Major allergen β -LG		Transport	0.619	0.948	0.952	0.989
Q2KIS7	Tetranectin	<i>CLEC3B</i>	Cell	0.909	0.88	0.889	0.978
Q95122	Monocyte differentiation antigen CD14	<i>CD14</i>	Immunity	1.078	1.014	0.983	0.962
Q3SZR3	α -1-acid glycoprotein	<i>ORM1</i>	Transport	1.132	0.913	0.733	0.94
Q9TRB9	Enterotoxin-binding glycoprotein PP20K		Unknown	1.073	1.05	0.874	0.933
Q3MHN5	Vitamin D-binding protein	<i>GC</i>	Transport	1.151	0.992	1.175	0.925
Q3ZCL0	Cysteine-rich secretory protein 3	<i>CRISP3</i>	Immunity	1.228	1.647	1.279	0.919
Q28049	α -LA	<i>LALBA</i>	Enzyme	0.726	0.77	1.118	0.917
P17690	β -2-glycoprotein 1	<i>APOH</i>	Coagulation	0.528	1.019	1.638	0.891
Q29RQ1	Complement component C7	<i>C7</i>	Immunity	NQ	1.38	0.99	0.89
Q0P569	Nucleobindin-1	<i>NUCB1</i>	Protein synthesis	1.252	0.998	0.945	0.881
Q9MZ06	Fibroblast growth factor-binding protein 1	<i>FGFBP1</i>	Cell	1.197	0.758	0.826	0.852
P02702	Folate receptor α	<i>FOLR1</i>	Protein synthesis	1.124	1.049	0.839	0.772
A8YXY3	15 kDa selenoprotein	<i>SEP15</i>	Protein synthesis	1	0.996	0.835	0.696
P10790	Fatty acid-binding protein, heart	<i>FABP3</i>	Transport	0.942	0.606	1.205	0.687

Continued

Table 2 (Continued). The ratios of common proteins and immune-related proteins between samples at different SCC levels (SCC1 = $<10^5$ cells/mL; SCC2 = 2.5×10^5 cells/mL; SCC3 = 4×10^5 cells/mL; SCC4 = 7.5×10^5 cells/mL; SCC5 = 10^6 cells/mL)

Uniprot ID	Protein name	Gene name	Biological function ¹	SCC2/ SCC1 ²	SCC3/ SCC1 ²	SCC4/ SCC1 ²	SCC5/ SCC1 ²
F1MUT3	Xanthine dehydrogenase/oxidase	<i>XDH</i>	Enzyme	1.341	0.528	1.079	0.638
P12799	Fibrinogen γ -B chain	<i>FGG</i>	Coagulation	1.207	1.308	1.678	0.547
Q0IIA4	Glycoprotein 2 (zymogen granule membrane)	<i>GP2</i>	Transport	0.871	0.551	0.906	0.457
P02672	Fibrinogen α chain	<i>FGA</i>	Coagulation	1.34	1.181	1.577	0.417
Q32PA1	CD59 molecule, complement regulatory protein	<i>CD59</i>	Immunity	NQ	0.26	1.35	0.35
Q9TUM6	Perilipin-2	<i>PLIN2</i>	Transport	0.906	0.214	0.778	0.262
P02668	Kappa-CN	<i>CSN3</i>	Transport	0.108	0.281	1.065	0.233
Q95114	Lactadherin	<i>MFGE8</i>	Cell	1.003	0.24	1.323	0.179
P18892	Butyrophilin subfamily 1 member A1	<i>BTN1A1</i>	Transport	0.829	0.135	1.375	0.156
Q27960	Sodium-dependent phosphate transport protein 2B	<i>SLC34A2</i>	Transport	1.153	0.107	1.135	0.106
P26201	Platelet glycoprotein 4	<i>CD36</i>	Cell	0.946	0.027	1.143	0.046
P12763	α -2-HS-glycoprotein	<i>AHSG</i>	Immunity	NQ	0.72	1.01	NQ
Q32PJ2	Apolipoprotein A-IV	<i>APOA4</i>	Immunity	0.99	NQ	NQ	NQ
P01888	β -2-microglobulin	<i>B2M</i>	Immunity	1.502	NQ	1.791	NQ
P81187	Complement factor B	<i>CFB</i>	Immunity	1.41	1.63	NQ	NQ

¹The biological functions were found using Uniprot (<http://www.uniprot.org/>) and DAVID (Dennis et al., 2003); Cell includes cell adhesion, cell mobility, cell growth, and cell apoptosis.

²Values more than one mean upregulated in milk with high SCC compared with milk with low SCC, whereas the values less than one mean downregulated in milk with high SCC compared with milk with low SCC. NQ = not quantified.

Figure 3. Log₂ ratio of protein concentration in milk with the highest SCC (SCC5 = 10^6 cells/mL) compared with milk with the lowest SCC (SCC1 = $<10^5$ cells/mL). iBAQ = intensity-based absolute quantification; ABCG2 = ATP-binding cassette subfamily G member 2; S100A8 = S100 calcium-binding protein A8; AMBP = α -1-microglobulin. Other abbreviations are defined in Table 2.

Figure 4. Relation between SCC and prostaglandin-H2 D-isomerase (PTGDS) concentration. (A) The relation between SCC and the concentration of PTGDS in 5 bulk-milk samples from 100 cows determined by liquid chromatography-tandem mass spectrometry. (B) The relation between SCC and the concentration of PTGDS in 5 bulk milk samples from 100 cows determined by ELISA. (C) The relation between SCC and the concentration of PTGDS in the milk from 28 individual cows determined by ELISA. iBAQ = intensity-based absolute quantification.

from cows with mastitis (Moyes et al., 2009; Huang et al., 2014). These changes in concentration of cellular function proteins were related to the immune response to mastitis infection (Genini et al., 2011). Because the complement regulatory protein CD59 can protect the mammary gland from overactivated complement mediated cell lysis and tissue damage (Bjorge et al., 1996), the decrease of CD59 in milk with high SCC (Figure 3) indicates its protective effect for the mammary gland during inflammation. Lactadherin (MFGE8) mediates phagocytosis of apoptotic epithelial cells. Downregulated MFGE8 can result in inefficient engulfment of dead cells by phagocytes (Boutet et al., 2004) and this delayed apoptosis would lead to necrotic cell death, which is believed to induce inflammation. Therefore, the decrease of cellular function proteins CD59 and MFGE8, as found in this study, indicates a stimulation of the immune response against inflammation.

Correlation Between PTGDS and SCC

The same linear correlation between PTGDS and SCC obtained from both LC-MS/MS results and ELISA (Figure 4A and 4B) shows the quantitative precision of the LC-MS/MS approach used in this study. The increase of PTGDS in the milk with high SCC was consistent with its increase in the milk from mastitis cows reported by Baeker et al. (2002). They hypothesized that the upregulation of PTGDS in milk was either the result of overexpression or damage of the blood-milk barrier caused by mastitis (Baeker et al., 2002). In addition, prostaglandins have been shown to induce chemokines resulting in infiltration of inflammatory cells, such as neutrophils, eosinophils, and macrophages (Aoki and Narumiya, 2012). This reflects that epithelial cells may produce PTGDS during mastitis to recruit the necessary immune cells through converting prostaglandin H2 to prostaglandin D2 (Figure 5), resulting in an increase in SCC. Although cathelicidins have been reported to be a biomarker for mastitis or subclinical mastitis, the correlation between cathelicidins and SCC was lower than the correlation between PTGDS and SCC (Supplemental Table S4; <http://dx.doi.org/10.3168/jds.2014-9279>). The nonlinear increase of cathelicidins was also reported by Smolenski et al. (2011), who suggested that it might be caused by a variation in neutrophil concentration and activity at different stages of subclinical mastitis. The higher correlation between PTGDS and SCC indicates that PTGDS may be a more suitable indicator for bulk-milk SCC. However, it seems to be not suitable for testing of milk from individual cows (Figure 4C). This inability to predict SCC of individual cow milk may be due to variation in the immune response between cows at

Figure 5. The role of prostaglandin-H2 D-isomerase (PTGDS) in the recruitment of SCC in the mammary gland from cows with mastitis inflammation (PGH2 = prostaglandin H2; PGD2 = prostaglandin D2), based on studies by Bonnefoy and Legrand (2000), Baeker et al. (2002), and Aoki and Narumiya (2012).

different stages of mastitis, which has been described before by Smolenski et al. (2011).

Bulk milk is used in the dairy industry for commercial production of dairy products. Therefore, a simple and easy test could be beneficial to distinguish bulk milk with high versus low SCC. Prostaglandin-H2 D-isomerase may thus be a potential candidate for such an easy test, which could for example be a color test or lab-on-a-chip assay. Such tests for enzymes in milk have previously already been developed for other enzymes such as the alkaline phosphatase assay for the validation of correct milk pasteurization (Rankin et al., 2010). According to the ELISA, the concentration of PTGDS in milk is above the detection limits of alkaline phosphatase assay, suggesting the feasibility of developing an easy color test. Such a test may be cheaper and simpler than advanced instruments such as the Fossomatic. In addition, PTGDS, in comparison with SCC, may provide additional insights in the mechanisms of mammary gland defense during infections if it would give additional information related to the stage of mastitis.

The effect of high SCC of cows on changes in their milk composition and subsequent effects on the quality of dairy products have been previously reported to be associated with increased proteolysis. For instance, milk with a high SCC level can lead to the development of off-flavors in pasteurized milk, UHT milk, yogurts, and cheese because of lipolysis and proteolysis (Maréchal et al., 2011). Elevated levels of FFA have been associated with rancidity, and extensive proteolysis in milk could

produce more hydrophobic peptides resulting in bitterness and astringency (Maréchal et al., 2011). Moreover, the shelf life of these dairy products were also shortened because of relatively high proteolytic activity in the milk with high SCC (Datta and Deeth, 2001; Santos et al., 2003; Maréchal et al., 2011). In addition, high SCC in milk also influences the coagulation properties and leads to increased moisture content and lower cheese yield (Barbano et al., 1991), resulting in economic losses. An easy and cheap test based on PTGDS could help separate milk with high SCC and thus avoid these negative influences on dairy product quality.

CONCLUSIONS

This is the first study investigating the qualitative and quantitative differences of the milk proteome from cows with different levels of SCC (range: 10^5 to 10^6 cells/mL). The results from this study indicate that the influence of high SCC is not related to the presence or absence of specific proteins but mainly involves the expression level of proteins. These results thereby contribute to better understanding of the influence of sub-clinical mastitis on the changes of the milk proteome, as well as the biological phenomena occurring in the mammary gland. The strong linear relation between PTGDS and SCC indicates that PTGDS may be a good candidate for developing a simple and easy test to evaluate bulk milk quality, by separating milk with high SCC to increase milk suitability for further milk processing. To understand the possible recruitment role

of PTGDS on SCC, further studies on the mechanism underlying the relation between PTGDS and SCC may be helpful.

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