J. Dairy Sci. 98:1–13
http://dx.doi.org/10.3168/jds.2014-9279

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, IGK 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 Log_{10}(SCC) (Koldeweij 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 (Schrick 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

Received December 23, 2014.
Accepted April 27, 2015.
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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 (2.25–2.75 $\times$ 10^5 cells/mL); SCC3 (4.8–5.3 $\times$ 10^5 cells/mL); SCC4 (7–8 $\times$ 10^5 cells/mL); and SCC5 (9.25–10.75 $\times$ 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 $\times$ 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 $\times$ 10^5 to 8.5 $\times$ 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× sample buffer (125 m*M* 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.