J. Dairy Sci. 100:1–13
https://doi.org/10.3168/jds.2016-11939

Ultra-performance liquid chromatography-quadrupole-time of flight mass spectrometry MS^E-based untargeted milk metabolomics in dairy cows with subclinical or clinical mastitis

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ABSTRACT

In this study, a novel metabolomics technique based on ultra-performance liquid chromatography-quadrupole-time of flight mass spectrometry in the MS^E mode was used to investigate the milk metabolomics of healthy, subclinical, and clinical mastitis cows, which were classified based on somatic cell count and presentation of clinical symptoms. Meanwhile, univariate and multivariate statistical analyses were performed to identify the significant differences across the 3 groups. Compared with healthy milk samples, less glucose, D-glycerol-1-phosphate, 4-hydroxyphenyllactate, L-carnitine, sn-glycero-3-phosphocholine, citrate, and hippurate were detected in the clinical mastitic milk samples, whereas less D-glycerol-1-phosphate, benzoic acid, L-carnitine, and *cis*-aconitate were found in the subclinical mastitic milk samples. Meanwhile, the milk concentration of arginine and Leu-Leu increased in both the clinical and subclinical mastitis groups. Besides, less 4-hydroxyphenyllactate, *cis*-aconitate, lactose, and oxoglutarate were detected in the clinical than the subclinical mastitic milk samples, whereas the abundance of some oligopeptides (Leu-Ala, Phe-Pro-Ile, Asn-Arg-Ala-Ile, and Val-Phe-Val-Tyr) increased by over 7.95-fold. Our results suggest that significant variations exist across healthy and mastitis cows. The current metabolomics approach will help in better understanding the pathobiology of mastitis, although clinical validation will be required before field application.

Key words: bovine mastitis, milk metabolome, ultra-performance liquid chromatography-quadrupole-time of flight mass spectrometry

INTRODUCTION

Milk is an important and complex biological fluid that contains a wide variety of nutrients, such as proteins, carbohydrates, lipids, and milk metabolites. Many factors can affect the composition of milk, such as stage of lactation, diet, seasonal variations, genetic variability, and cow health status (e.g., mastitis and ketosis; Jakob and Puhan, 1992; Enjalbert et al., 2001; Lock and Garnsworthy, 2003; Klein et al., 2010; Le Maréchal et al., 2011; Trenerry et al., 2013). The changes of milk composition affect the technological and nutritional properties of the milk (Politis and Ng-Kwai-Hang, 1988; Wedholm et al., 2006).

Mastitis is one of the most common diseases in dairy cows, causing huge economic losses to the worldwide dairy industry (Janzen, 1970; Gomes et al., 2016). It is an inflammation of the mammary gland or the udder, which is usually caused by pathogenic microorganisms or physical and chemical stimuli such as harmful toxins/chemicals or trauma. Mastitis has 2 forms: subclinical and clinical. Subclinical mastitis is a mastitis inflammation with no visual indicators in milk or mammary gland, but characterized by an increase in the milk SCC. The milk quality is likely affected, accompanied by a decrease in milk production. Inflammation may occur with or without any intramammary pathogen present. In contrast, clinical mastitis is characterized by an elevated SCC in milk together with visual signs of inflammation such as clumpy, watery, bloody, or yellowish milk, as well as swelling, redness, and pain in the udder. It is also likely that pathogens are found in the milk of cows with clinical mastitis (Moyes et al., 2009; Sundekilde et al., 2013b). In both cases, the properties of milk, SCC, and profile of milk metabolites will be altered (Batavani et al., 2007; Le Maréchal et al., 2011; Sundekilde et al., 2013a). The level of SCC has been used as a cow mastitis diagnostic indicator; an SCC below 100,000 cells/mL with no bacterial growth cultivated from the milk sample indicates a healthy quarter, whereas the level of over 200,000 cells/mL suggests the

Received August 30, 2016.
Accepted December 16, 2016.
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presence of infection (Pyörälä, 2003). Recently, an SCC cutoff of 100,000 cells/mL has been suggested for differentiating infected from noninfected mammary glands (Schwarz et al., 2010; dos Reis et al., 2011). Schwarz et al. (2010) showed that an SCC above 100,000 cells/mL is normally related to inflammatory processes inside the mammary gland. The accepted SCC threshold for bulk milk varies in different countries. For example, the cutoff levels for Germany, the European Union, China, Canada, and the United States are 100,000, 400,000, 500,000, 500,000, and 750,000 cells/mL, respectively (Olechnowicz and Jaśkowski, 2012; Li et al., 2014). Furthermore, the SCC is related to an influx of milk metabolites such as fat, free AA, organic acids, and other low molecular weight metabolites (Sundekilde et al., 2013b). Some important metabolites, including uracil, lactate, acetate, isoleucine, butyrate, and hippurate, are correlated with the SCC level (Melzer et al., 2013; Sundekilde et al., 2013b). These milk metabolites may be originated from milk enzymatic reactions, secreted by the mammary epithelial cells into the milk, derived from microorganisms in raw milk, or both. In any case, the milk metabolite profile directly reflects the physiological and health status of the mammary gland.

Metabolomics is a new branch of "-omics" science, which studies the composition, relative abundance, dynamics, and interactions among metabolites in a given organism and biological system in response to various stimuli or interventions (Osorio et al., 2012). It has been used for characterizing the metabolic profiles of the test and control groups, as well as subjects of different health statuses. For example, the approach has been widely used in analyzing the relationship between milk metabolites and mastitis. Hettinga et al. (2008) employed solid-phase microextraction coupled with GC-MS technology to investigate volatile metabolites in mastitic cow milk samples and found that the profile of milk volatile compounds significantly varied between infections caused by different pathogens. High-throughput and sensitive liquid chromatography (**LC**)-MS is a powerful technology for global metabolic profiling of complex biological samples. It can detect most metabolites including low and high molecular weight compounds as well as hydrophilic and hydrophobic compounds and identify biomarkers (Putri et al., 2013). By applying LC-MS, Mansor (2012) compared the milk metabolite profiles of healthy and mastitis cows infected by *Escherichia coli* and *Staphylococcus aureus*. A total of 1,105 metabolites were detected in the milk samples using the MzMine software (version 2.0; http://mzmine.sourceforge.net/index.shtml), with an increase in di- and tri-peptides in the mastitic milk samples compared with the healthy ones. Besides, pathway analyses showed that the level of arachidonic acid-, arginine-, and galactose-related metabolites were elevated in mastitic milk samples. Furthermore, the LC-MS-based metabolomics approach was also used to monitor the changes in the milk metabolome along the course of *Streptococcus uberis* infection (Thomas, 2015).

However, molecular methods have recently revealed the presence of mixed bacterial infections in some bovine mastitis cases (Kuang et al., 2009; Koskinen et al., 2010; Oikonomou et al., 2012). Additionally, Bhatt et al. (2012) finds that subclinical mastitis is not merely caused by a single pathogenic species of bacteria but a blend of several microbes. And bovine mastitis normally contains 2 forms: subclinical and clinical mastitis. Thus, in this study, the ultra-performance liquid chromatography-quadrupole-time of flight mass spectrometry (**UPLC-Q-TOF MS**) in the MS^E mode coupled with univariate and multivariate statistics was used to compare the milk metabolome differences of healthy, subclinical, and clinical mastitic cows in field conditions. Our study has chosen to use the UPLC-Q-TOF MS^E, as it has been shown to be of higher sensitivity than other metabolomics techniques such as nuclear magnetic resonance spectroscopy (Boudonck et al., 2009; Sundekilde et al., 2011). The ultimate goal of this study was to identify milk metabolic signatures and biomarkers that were specific to cow mastitis. Such data will further our understandings of mastitis-related metabolic changes and provide information on possible measures for the enhancement of animal management, diagnosis and treatment of subclinical and clinical mastitis.

MATERIALS AND METHODS

Ethics Statement

This animal experiment was approved by the Ethics Committee of Inner Mongolia Agricultural University. The collection of cow milk samples was permitted by the owner of the farm.

Collection of Milk Samples

Milk samples were collected from Chinese Holstein cows at a farm near Hohhot, China. All Holstein cows were 3 to 6 yr and housed in an open loose farm, fed with TMR according to standard practice, and milked with an automatic milking system twice per day. The information of parity, lactation stage, and TMR ration of selected cows is given in Table 1. Milk samples were collected in sterile centrifuge tubes and stored on ice during transportation to the laboratory. One aliquot of these milk samples was analyzed for SCC in the laboratory with a Bentley FTS/FCM400 Combi Instrument