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 Puhon, 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

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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

(Chaska, MN). Another aliquot was skimmed by centrifugation at $4,500 \times g$ for 10 min at 4°C, and the skim milk was frozen at -80°C for further analysis. Although the International Dairy Federation originally suggested a 500,000 cells/mL reference value for a healthy quarter and recommended using an SCC cutoff of 200,000 cells/mL to define mastitis in composite bovine milk, it has been generally recognized that the SCC range of sub-clinical mastitis varies widely (International Dairy Federation, 1971, 2013). Moreover, the SCC threshold of 100,000 cells/mL was confirmed to be appropriate for differentiating infected from noninfected mammary glands (Schwarz et al., 2010; dos Reis et al., 2011). Thus, in the study, the cow udder status was defined according to both SCC and presentations of clinical symptoms. Milk samples were divided into 3 groups: 20 milk samples from 20 healthy cows (SCC <100,000 cells/mL; with no clinical signs of inflammation, e.g., swelling, redness, and pain in the udder, and presence of clots in milk); 20 milk samples from 20 subclinical mastitis cows (SCC between 100,000 and 500,000 cells/mL; without obvious clinical mastitis signs); and another 20 milk samples obtained from 20 clinical mastitis cows (with obvious clinical signs of inflammations including swelling, redness, and pain in the udder, and clots in milk; SCC >500,000 cells/mL). The milk sample SCC is shown in Table 1.

Milk Samples Preparation for UPLC-Q-TOF MS^E Analysis

Prior to UPLC-Q-TOF MS^E, the 60 skim milk samples were thawed and thoroughly vortex mixed. An aliquot of 2 mL of each milk sample was added to 14 mL of acetonitrile (LC-MS grade quality, Thermo Fisher Scientific, Waltham, MA), mixed by vortexing for 2 min and ultrasonic processing for 5 min. Next, the sample was centrifuged at $12,000 \times g$ for 15 min at 4°C to remove large biomolecules such as proteins. The supernatant was then vacuum concentrated and dissolved in 500 µL of 40% (vol/vol) acetonitrile. Before transferring to autosampler vials, the samples were filtered through a 0.22-µm MS nylon syringe filter.

UPLC-Q-TOF MS^E Analysis

The milk metabolites analysis was performed using an Acquity UPLC system (Waters Corp., Milford, MA) coupled with a Waters Q-TOF Premier mass spectrometer equipped with an electrospray ionization (ESI) source, which was operated in both positive and negative ion modes (ESI⁺ and ESI⁻).

Chromatographic separations were performed on an Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7

Table 1. Information on healthy cows and cows with subclinical and clinical mastitis that provided milk samples

Sample group ¹	Cow parity	Cow lactation stage (mo)	Condition of collected milk	Udder status	Milk SCC (×10 ³ cells/mL) average and range	TMR (kg/d)			
						Hay	Corn silage	Mixed concentrate	Sodium bicarbonate
H	2-4	3-7	—	No	47.3 ± 19.6, 18-80	5	26	10	0.1
SM	2-4	3-7	—	No	301.5 ± 75.1, 144-435	5	26	10	0.1
CM	2-4	3-7	Clotted	Swelling or redness	2,019.7 ± 741, 583-3,282	4	26	9	0.1

¹H, SM, and CM represent healthy, subclinical mastitic, and clinical mastitic groups, respectively; the milk SCC is expressed as mean ± SD.

μm ; Waters Corp.). Each milk sample (10 μL) was injected onto the column at a flow rate of 0.45 mL/min, and the column was maintained at 40°C. As for ESI⁺, the mobile phase consisted of water containing 0.1% formic acid (solution A) and acetonitrile containing 0.1% formic acid (solution B), and the elution gradient was as follows: 2% B for 2 min; 2 to 7% B over 2.0 to 2.5 min; 7 to 50% B over 2.5 to 9.0 min; 50 to 85% B over 9.0 to 13.0 min; 85 to 95% B over 13.0 to 19.5 min; 95% B for 0.5 min; then return to 2% B for 2.0 min. As for ESI⁻, the mobile phase consisted of water containing 0.1% ammonium hydroxide (solution A) and acetonitrile (solution B), and the elution gradient was as follows: 10% B for 2 min; 10 to 15% B over 2.0 to 2.5 min; 15 to 50% B over 2.5 to 9.0 min; 50 to 85% B over 9.0 to 13.0 min; 85 to 95% B over 13.0 to 19.5 min; 95% B for 0.5 min; then return to 2% B for 2.0 min.

The mass data were acquired in both positive and negative ion modes. The analytical parameters were set as follows: capillary voltage, 3 kV (+) and 2.5 kV (-), respectively; sample cone voltage, 40 V; source temperature, 100°C; desolvation gas temperature, 350°C; desolvation gas (nitrogen) flow, 800 L/h (+) and 600 L/h (-), respectively; cone gas (nitrogen) flow, 50 L/h. The mass spectrometer was operated in both MS and MS^E modes. With the MS^E experiment, both precursor exact ion mass in low energy and fragment ion information in high energy were acquired in one run. The MS collision energy was 6 eV for precursor ions, and MS^E collision energy was set at 20 to 45 eV for product ions. The full-scan range was from 50 to 1,000 m/z .

To ensure mass reproducibility and accuracy, leucine-enkephalin was used as the lock mass for positive ion mode (556.2771 [M+H]⁺) and negative ion mode (554.2615 [M-H]⁻). The concentration was 2 ng/mL, the flow rate was 30 $\mu\text{L}/\text{min}$, and the lock-spray frequency was 10 s. The quadrupole-time of flight MS data were collected in continuum mode and processed by MassLynx and Progenesis QI software (Waters Corp.).

Data Processing and Statistical Analyses

The raw data files created by UPLC-Q-TOF MS^E were imported into the Progenesis QI software for peak alignment, peak picking, and deconvolution. After being recognized and aligned, the individual ion intensities were normalized for all compounds. Then the data sets including peak number (retention time- m/z pair), sample name and normalized ion intensities were exported to the EZinfo software (Waters Corp.) for multivariate statistical analysis such as principal component analysis (PCA) and orthogonal projection to latent structures-discriminant analysis (OPLS-DA). Before multivariate statistical analysis, all data were mean-

centered and Pareto-scaled. In addition, the t -test and fold change analysis were performed using the Progenesis QI to identify the significantly different metabolites between sample groups.

Three criteria were used for defining differential metabolites between groups: minimum fold change (≥ 2), ANOVA P -value (≤ 0.05), and variable importance value (VIP) calculated in the OPLS-DA model (≥ 1). The qualified filtered variables were further analyzed and identified.

Identification of Metabolites and Metabolic Pathway Analysis

The differential metabolites were identified using the Progenesis QI software based on m/z , retention time, and fragment information. The software was set to automatically search the selected databases such as HMDB (<http://www.hmdb.ca/>), ECMDDB (<http://www.ecmdb.ca/>), YMDB (<http://www.ymdb.ca/>), and METLIN (<https://metlin.scripps.edu/>) to confirm the identity of the candidate molecules. The mass error for database search was 20 ppm or less. The differential metabolites were further analyzed using the MetaboAnalyst 3.0 web-server (<http://www.metaboanalyst.ca/>) combined with the KEGG PATHWAY database (<http://www.kegg.jp/kegg/pathway.html>).

RESULTS

UPLC-Q-TOF MS^E Metabolite Profiling of Milk Samples

In this study, the UPLC-Q-TOF MS^E technology was used to investigate the metabolite profiles of milk samples collected from cows of different healthy statuses (healthy, subclinical, or clinical mastitis). Typical positive and negative base peak intensity chromatograms of milk collected from healthy, subclinical mastitis, and clinical mastitis cows are shown in Supplemental Figures S1 and S2, respectively (<https://doi.org/10.3168/jds.2016-11939>). The typical base peak intensity chromatogram profile of the healthy and subclinical mastitic milk samples shared a very high similarity, although largely different from that of the clinical mastitic one in terms of number of peaks, retention time, and peak area. However, only several peaks and their peak intensities were different between the healthy and subclinical mastitic milk profiles for both the ESI⁺ and ESI⁻ chromatograms. Our results indicate that apparent differences existed between the milk metabolome of the 3 groups; the differences were more obvious between the healthy and clinical mastitis group than that between the healthy and subclinical mastitis one.

Metabolomics Data Analysis

To visualize the metabolic differences between the 3 milk groups, an unsupervised PCA was performed based on the metabolome generated in the positive and negative modes (Figure 1). In both PCA score plots, symbols representing the 3 milk sample groups clearly separated and distributed in different quadrants, indicating clear differences existed between their metabolic profiles. The data were further processed by a supervised OPLS-DA to identify the differential metabolites. The score plot and S-plots in both positive and negative ion modes are shown in Figures 2 and 3, respectively. The parameters R^2Y and Q^2 were all above 0.79 for all 6 models, indicating a high fitness and predicting ability. In the S-plots, each dot represents a variable, and the farther the distance from the origin, the higher confidence and magnitude of each variable contribution to the group difference. Those dots locating at the top right corner and the lower left corner of the S-plots were the most discriminating variables having a VIP of ≥ 1 (as shown by the rectangles in Figures 2 and 3).

Differential Metabolites and Their Identification

A total of 1,872 (1,289 and 583 by positive and negative ion mode, respectively) metabolites from all 60 milk samples were detected. Among them, 91 differential metabolites, with P -values of ≤ 0.05 , VIP of ≥ 1 , and minimum fold changes of ≥ 2 , were detected between samples of healthy and mastitis cows. The milk metabolome were analyzed with OPLS-DA in a pair-wise manner. Differential metabolites between the

milk metabolomes of the healthy versus clinical mastitis, healthy versus subclinical mastitis, and clinical mastitis versus subclinical groups are listed in Tables 2 to 3, Tables 4 to 5, and Tables 6 to 7, respectively. Among these significantly different metabolites, D-glycerol-1-phosphate, L-carnitine, 5-methoxytryptophan, and indolylacryloylglycine decreased, whereas Arg and Leu-Leu increased in the milk samples from both the subclinical and clinical mastitis groups compared with the healthy one. Besides, less 4-hydroxyphenyllactate was detected in the milk samples from clinical mastitis cows.

Metabolic Pathway Analysis

MetaboAnalyst 3.0 was used to investigate and map the metabolic differences among healthy, subclinical, and clinical mastitis cows, as reflected by the milk metabolites that significantly differed between the 3 groups. The major metabolic pathways include those directly involved in the carbohydrate and energy, lipid, and protein metabolisms. Seven metabolic pathways that showed apparent variations between the subclinical and clinical mastitis groups are shown in Table 8. Specifically, in the clinical mastitis cow milk, most differential identified compounds that were involved in the carbohydrate and energy and lipid metabolisms were less abundant, whereas mostly more small peptides and AA were detected. In addition, some metabolites involved in nucleotide metabolism, vitamin metabolism, biosynthesis of secondary metabolites, and unknown pathways showed apparent variations between groups, which are shown in Tables 2 to 7.

Figure 1. Principal component analysis score plots of the milk metabolome of 3 milk groups in positive [electrospray ionization, ESI (+)] and negative [ESI (-)] ion modes. Milk samples from healthy, subclinical, and clinical mastitis cow groups are represented by H, SM, and CM, respectively. Color version available online.

DISCUSSION

Bovine mastitis has drawn wide attention, as it causes major economic loss to the dairy industry. Therefore, accurate and rapid mastitis diagnosis and effective control are crucial to the industry. Some previous studies have applied the untargeted metabolomics technique to identify biomarkers that are specific to typical bovine mastitis causative pathogens, including *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus uberis* (Mansor, 2012; Thomas, 2015). However, in field conditions, bovine mastitis is often caused by diverse pathogens, and depending on the

degree of mammary gland inflammation, the disease is classified as subclinical and clinical mastitis. The identification of potential bovine mastitis biomarkers suitable for field conditions will provide new insights into the treatment of bovine mastitis. Thus, we used UPLC-Q-TOF MS^E to generate and compare the milk metabolite profiles of healthy cows and those suffering from subclinical and clinical mastitis. Our PCA results revealed apparent differences between the milk metabolite profiles of the 3 groups. By OPLS-DA and *t*-test, we identified some candidate cow milk biomarkers and pathways that were linked with the mastitis disease status.

Figure 2. Orthogonal partial least squares-discriminant analysis score plots and S-plots showing pairwise comparison between groups in positive ion mode. On the score plots, the milk metabolome of each sample is represented by one symbol; samples from healthy, subclinical, and clinical mastitis cow groups are represented by H, SM, and CM, respectively. R^2Y and Q^2 are fitness power and predicting power, respectively. On the S-plots, each dot corresponds to a single metabolite. Metabolites with variable importance value ≥ 1 are enclosed by a rectangle. Color version available online.

Figure 3. Orthogonal partial least squares-discriminant analysis score plots and S-plots showing pairwise comparison between groups in negative ion mode. On the score plots, the milk metabolome of each sample is represented by one symbol; samples from healthy, subclinical, and clinical mastitis cow groups are represented by H, SM, and CM, respectively. R^2Y and Q^2 are fitness power and predicting power, respectively. On the S-plots, each dot corresponds to a single metabolite. Metabolites with variable importance value ≥ 1 are enclosed by a rectangle. Color version available online.

Specifically, several metabolic differences were observed in milk samples collected from mastitis cows, as compared with the healthy ones. Metabolites of the carbohydrate and energy metabolism were significantly different between milk from the healthy and clinical mastitis cows but not the subclinical ones. It was previously reported that this group of metabolites, such as glucose, citrate, (S)-malate, *cis*-aconitate, and oxoglutarate, decreased at the peak of infection (Thomas, 2015). Meanwhile, less milk carbohydrate and energy related metabolites were detected in the clinical but not subclinical mastitis cows. As mastitis progresses, the pathogens might have used up more of these

compounds for their activities and growth. These differential metabolites may be further explored for their potential as biomarkers for diagnosis between these 2 bovine mastitis stages. As observed in some studies (Marschke and Kitchen, 1984; Mansor, 2012; Thomas, 2015), the concentration of glucose was reduced in the milk samples from clinical mastitis cows. The exact reason for such change is unknown, but one possible explanation is the restriction of blood flow to the infected mammary gland, resulting in a limited blood glucose supply to the secretory cells (Linzell and Peaker, 1971). Another hypothesis is that glucose is transported out of the milk by paracellular pathway to maintain os-

Table 2. Significantly differing metabolites between milk samples from healthy and clinical mastitis cows, positive ion mode analysis

Row <i>m/z</i>	Retention time (min)	Identity of differential metabolites	Molecular formula	Metabolite type ¹	Fold change	<i>P</i> -value
Reduced in clinical mastitis group						
245.0788	9.26	Uridine	C ₉ H ₁₂ N ₂ O ₆	NT	3.64	2.69 × 10 ⁻⁵
173.0239	6.31	D-Glycerol 1-phosphate	C ₃ H ₆ O ₆ P	LL	2.91	2.98 × 10 ⁻⁵
175.0212	6.31	Citrate	C ₆ H ₈ O ₇	C/E	2.82	3.79 × 10 ⁻⁵
181.0758	4.91	Glucose	C ₆ H ₁₂ O ₆	C/E	4.16	0.00269
199.0868	4.91	5-Methoxytryptophan	C ₁₂ H ₁₄ N ₂ O ₃	0	3.20	0.00273
163.0386	7.57	4-Hydroxyphenylpyruvate	C ₉ H ₈ O ₄	AA	2.14	0.00279
184.0735	4.91	Phosphocholine	C ₅ H ₁₄ NO ₄ P	LL	5.66	0.00484
135.0286	9.42	(S)-Malate	C ₄ H ₆ O ₅	C/E	2.99	0.0094
258.1127	5.12	sn-glycero-3-Phosphocholine	C ₈ H ₂₀ NO ₆ P	LL	2.31	0.01023
301.1412	12.08	Alpha-CEHC ²	C ₁₆ H ₂₂ O ₄	Vit	2.06	0.01127
187.0965	4.53	Isopropyl β-D-glucoside	C ₉ H ₁₈ O ₆	0	2.02	0.01152
209.0708	4.91	Indolylacryloylglycine	C ₁₃ H ₁₂ N ₂ O ₃	SM	3.70	0.01463
246.1707	4.53	2-Methylbutyrylcarnitine	C ₁₂ H ₂₃ NO ₄	LL	2.01	0.01974
165.0547	1.28	4-Hydroxyphenyllactate	C ₉ H ₁₀ O ₄	AA/SM	3.19	0.00312
162.1123	0.57	L-Carnitine	C ₇ H ₁₅ NO ₃	LL	2.07	0.04857
Increased in clinical mastitis group						
118.0866	1.30	L-Valine	C ₅ H ₁₁ NO ₂	AA	10.24	5.87 × 10 ⁻⁶
527.2858	7.18	Val-Tyr-Leu-Arg	C ₂₆ H ₄₃ N ₇ O ₆	PP	96.80	1.14 × 10 ⁻⁵
211.1449	7.42	Isoleucyl-proline	C ₁₁ H ₂₀ N ₂ O ₃	PP	28.11	3.48 × 10 ⁻⁵
799.48	7.42	PI [18:3(6Z,9Z,12Z)/15:1(9Z)] ³	C ₄₂ H ₇₃ O ₁₃ P	LL	26.45	6.5 × 10 ⁻⁵
239.1512	4.86	Gln-Lys	C ₁₁ H ₂₂ N ₄ O ₄	PP	13.44	0.00021
263.1398	5.34	Tyrosyl-valine	C ₁₄ H ₂₀ N ₂ O ₄	PP	7.09	0.00044
132.1025	2.44	L-Isoleucine	C ₆ H ₁₄ NO ₂	AA	9.21	0.00087
342.2381	6.89	Leu-Pro-Ile	C ₁₇ H ₃₁ N ₃ O ₄	PP	16.84	0.00152
116.0709	1.02	L-Proline	C ₅ H ₉ NO ₂	AA	3.11	0.00299
227.1763	5.21	Leu-Leu	C ₁₂ H ₂₄ N ₂ O ₃	PP	2.48	0.00198
136.075	1.27	Dopamine	C ₈ H ₁₁ NO ₂	AA/SM	2.18	0.01207

¹AA = AA metabolites; PP = small peptides; C/E = carbohydrate and energy metabolites; LL = lipid metabolites; Vit = vitamins and related metabolites; NT = nucleotide metabolites; SM = biosynthesis of secondary metabolites; 0 = unknown pathway.

²Alpha-CEHC = 2,5,7,8-tetramethyl-2-(2'-carboxyethyl)-6-hydroxychroman.

³PI = phosphatidylinositol.

Table 3. Significantly differing metabolites between milk samples from healthy and clinical mastitis cows, negative ion mode analysis

Row <i>m/z</i>	Retention time (min)	Identity of differential metabolites	Molecular formula	Metabolite type ¹	Fold change	<i>P</i> -value
Reduced in clinical mastitis group						
564.4076	4.42	PC (21:0/0:0) ²	C ₂₉ H ₆₀ NO ₇ P	LL	3.06	9.10 × 10 ⁻¹¹
178.05	4.31	Hippurate	C ₉ H ₉ NO ₃	AA	3.87	6.31 × 10 ⁻⁷
195.0659	0.39	2-Hydroxy-3-(4-methoxyphenyl)propanoic acid	C ₁₀ H ₁₂ O ₄	0	64.4	6.80 × 10 ⁻⁷
296.1016	0.67	2-Methylguanosine	C ₁₁ H ₁₅ N ₅ O ₅	NT	7.37	4.47 × 10 ⁻⁵
240.1253	0.84	N-(3-oxo-octanoyl)-homoserine lactone	C ₁₂ H ₁₉ NO ₄	0	3.71	4.82 × 10 ⁻⁶
362.055	0.67	Guanosine monophosphate	C ₁₀ H ₁₄ N ₅ O ₈ P	NT	6.75	0.00085
285.2284	9.17	Retinol	C ₂₀ H ₃₀ O	Vit	2.94	0.00179
Increased in clinical mastitis group						
409.3057	11.99	N-oleoyl glutamine	C ₂₃ H ₄₂ N ₂ O ₄	0	13.4	2.63 × 10 ⁻¹¹
217.12	4.86	Leu Ser	C ₉ H ₁₈ N ₂ O ₄	PP	43.21	4.98 × 10 ⁻⁹
215.068	5.78	Thr Asp	C ₈ H ₁₄ N ₂ O ₆	PP	27.94	5.85 × 10 ⁻⁹
435.3101	11.97	Ile Ile Ile Pro	C ₂₃ H ₄₂ N ₄ O ₅	PP	39.8	1.86 × 10 ⁻⁸
329.0295	4.43	Inosinic acid (inosine monophosphate)	C ₁₀ H ₁₃ N ₄ O ₈ P	NT	Infinity	1.40 × 10 ⁻⁷
433.2954	11.51	N-stearoyl glutamine	C ₂₃ H ₄₄ N ₂ O ₄	0	29.4	2.37 × 10 ⁻⁷
540.3351	11.97	Arg Thr Lys Arg	C ₂₂ H ₄₅ N ₁₁ O ₆	PP	23.2	4.11 × 10 ⁻⁷
556.3235	11.97	Leu Arg Asn Arg	C ₂₂ H ₄₃ N ₁₁ O ₆	PP	28.56	5.40 × 10 ⁻⁷
155.0984	0.88	L-Arginine	C ₆ H ₁₄ N ₄ O ₂	AA	2.66	2.69 × 10 ⁻⁵
485.317	8.68	Arg Arg Arg	C ₁₈ H ₃₈ N ₁₂ O ₄	PP	5.46	4.31 × 10 ⁻⁵

¹AA = amino acid metabolites; PP = small peptides; LL = lipid metabolites; Vit = vitamins and related metabolites; NT = nucleotide metabolites; 0 = unknown pathway.

²PC = phosphatidylcholine.

Table 4. Significantly differing metabolites between milk samples from healthy and subclinical mastitis cows, positive ion mode analysis

Row <i>m/z</i>	Retention time (min)	Identity of differential metabolites	Molecular formula	Metabolite type ¹	Fold change	<i>P</i> -value
Reduced in subclinical mastitis group						
218.1386	6.72	O-propanoyl-carnitine	C ₁₀ H ₁₉ NO ₄	LL	2.61	0.00037
173.0228	6.39	D-Glycerol 1-phosphate	C ₃ H ₉ O ₆ P	LL	2.15	0.00054
105.0311	4.38	Benzoic acid	C ₇ H ₆ O ₂	AA	2.27	0.00096
162.1115	0.57	L-Carnitine	C ₇ H ₁₅ NO ₃	LL	2.75	0.00556
199.0863	4.91	5-Methoxytryptophan	C ₁₂ H ₁₄ N ₂ O ₃	0	3.74	0.00631
230.2475	10.12	Xestoaminol C	C ₁₄ H ₃₁ NO	0	2.03	0.0108
209.0709	4.91	Indolylacryloylglycine	C ₁₃ H ₁₂ N ₂ O ₃	SM	2.7	0.018
Increased in subclinical mastitis group						
701.4942	5.79	1,2-dihexadecanoyl-sn-glycero-3-phosphosulfocholine	C ₃₉ H ₇₇ O ₈ PS	0	2.87	4.99 × 10 ⁻⁷
425.2122	12.87	Lys Asp Tyr	C ₁₉ H ₂₈ N ₄ O ₇	PP	3.96	8.45 × 10 ⁻⁸
213.0744	12.89	L-Arginine	C ₆ H ₁₄ N ₄ O ₂	AA	2.66	0.00019
209.1648	5.74	Leucyl-Leucine	C ₁₂ H ₂₄ N ₂ O ₃	PP	2.43	0.00049

¹AA = amino acid metabolites; PP = small peptides; LL = lipid metabolites; SM = biosynthesis of secondary metabolites; 0 = unknown pathway.

motric balance between extracellular environment and the milk, as Na⁺ and Cl⁻ increase in milk during mastitis (Marschke and Kitchen, 1984). Besides, the low glucose may have resulted from the gathering of cells at the infected sites, and they used up the glucose in the localized area. An adequate level of plasma glucose is essential for maintaining milk secretion, and thus a reduced glucose level can result in cessation of milk secretion and decreased milk yield in mastitis (Rook et al., 1965). In addition, the levels of some TCA cycle intermediates such as citrate, (S)-malate, *cis*-aconitate, and oxoglutarate also dropped significantly in the clinical mastitic milk samples, which was also reported by Thomas (2015), suggesting that the TCA cycle is downregulated in bovine mastitis.

Lipids, such as triacylglycerols, diacylglycerols, monoacylglycerols, free fatty acids, phospholipids, and sterols, are important milk components (MacGibbon and

Taylor, 2006). During mastitis, the extent of lipolysis increases and this leads to an elevated level of free fatty acids in milk (Randolph and Erwin, 1974; Salih and Anderson, 1979; Atroshi et al., 1989; Hunt et al., 2013). However, we did not observe such a phenomenon. Possibly, the discrepancy was due to the sample extraction and the chromatographic separation (Thomas et al., 2016). The free fatty acids and lipid compounds should be separately analyzed by GC and lipidomic methods in future studies.

However, similar to Thomas (2015), we observed decreases in the levels of sn-glycero-3-phosphocholine, D-glycerol-1-phosphate, and glycerol phospholipids in the milk samples of clinical and subclinical mastitis cows. The milk content of glycerol phospholipids decreases as mastitic infection progresses, possibly because the cell membrane of mammary cells could have been destroyed during mastitis or are hydrolyzed by phospholipases and

Table 5. Significantly differing metabolites between milk samples from healthy and subclinical mastitis cows, negative ion mode analysis

Row <i>m/z</i>	Retention time (min)	Identity of differential metabolites	Molecular formula	Metabolite type ¹	Fold change	<i>P</i> -value
Reduced in subclinical mastitis group						
211.1943	4.86	Capryloylcholine	C ₁₃ H ₂₈ NO ₂ ⁺	LL	Infinity	0
219.0819	1.28	5-Hydroxy-L-tryptophan	C ₁₁ H ₁₂ N ₂ O ₃	AA	416.56	1.11 × 10 ⁻¹⁶
435.1013	11.97	Cys Cys Asp Pro	C ₁₅ H ₂₄ N ₄ O ₇ S ₂	PP	3.30	1.12 × 10 ⁻⁷
207.06	1.28	L-4-Hydroxyglutamine	C ₅ H ₁₀ N ₂ O ₄	0	2.6	6.65 × 10 ⁻⁷
205.1192	0.72	Dimethylglycine	C ₄ H ₉ NO ₂	AA	30.06	7.14 × 10 ⁻⁷
497.3629	3.88	7,11-Dioxolanost-8-en-3-yl acetate	C ₃₂ H ₅₀ O ₄	0	2.6	9.47 × 10 ⁻⁷
173.01	12.89	<i>cis</i> -Aconitate	C ₆ H ₆ O ₆	C/E	2.18	0.00028
Increased in subclinical mastitis group						
255.0035	1.05	2-Thiouracil	C ₄ H ₄ N ₂ OS	0	4.95	4.93 × 10 ⁻¹³
155.0984	0.88	L-Arginine	C ₆ H ₁₄ N ₄ O ₂	AA	6.72	5.81 × 10 ⁻¹³
330.2341	8.73	Ile Val Val	C ₁₆ H ₃₁ N ₃ O ₄	PP	3.58	0.00058

¹AA = amino acid metabolites; PP = small peptides; C/E = carbohydrate and energy metabolites; LL = lipid metabolites; 0 = unknown pathway.

Table 6. Significantly differing metabolites between milk samples from subclinical and clinical mastitis cows, positive ion mode analysis

Row <i>m/z</i>	Retention time (min)	Identity of differential metabolites	Molecular formula	Metabolite type ¹	Fold change	<i>P</i> -value
Reduced in clinical mastitis group						
566.4276	5.54	PC (21:0/0:0) ²	C ₂₉ H ₆₀ NO ₇ P	LL	2.43	8.56 × 10 ⁻⁵
216.0639	4.96	2-Methylhippuric acid	C ₁₀ H ₁₁ NO ₃	AA	3.81	8.88 × 10 ⁻⁵
261.0375	0.55	Glucose 1-phosphate	C ₆ H ₁₃ O ₉ P	C/E	2.76	0.00016
119.0477	1.25	2-Methylbenzoic acid	C ₈ H ₈ O ₂	SM	2.64	0.00022
365.1052	0.59	D-Lactose	C ₁₂ H ₂₂ O ₁₁	C/E	2.52	0.00057
129.017	12.89	Oxoglutarate	C ₅ H ₆ O ₅	C/E	2.67	0.00104
139.0016	12.89	<i>cis</i> -Aconitate	C ₆ H ₆ O ₆	C/E	2.63	0.00112
165.054	1.25	4-Hydroxyphenyllactate	C ₉ H ₁₀ O ₄	AA/SM	2.69	0.00137
185.0808	12.89	Phosphorylcholine	C ₁₅ H ₄₂ N ₃ O ₇ P	LL	2.53	0.00158
377.145	4.73	Riboflavin (vitamin B ₂)	C ₁₇ H ₂₀ N ₄ O ₆	Vit	3.16	0.00657
Increased in clinical mastitis group						
237.1452	5.54	Asn Arg Ala Ile	C ₁₉ H ₃₆ N ₈ O ₆	PP	Infinity	3.54 × 10 ⁻¹³
527.2859	7.27	Val Phe Val Tyr	C ₂₈ H ₃₈ N ₄ O ₆	PP	45.6	3.69 × 10 ⁻⁵
410.2501	6.94	9,10-dihydroxy-Octadecanedioic acid	C ₁₈ H ₃₄ O ₆	0	13.1	0.00011
376.2197	6.79	Phe Pro Ile	C ₂₀ H ₂₉ N ₃ O ₄	PP	7.95	0.00037
173.0804	5.61	2-Octenedioic acid	C ₈ H ₁₂ O ₄	0	8.89	0.00087
239.1513	4.93	Gln Lys	C ₁₁ H ₂₂ N ₄ O ₄	PP	4.11	0.00148
360.1919	5.39	Pro Pro Phe	C ₁₉ H ₂₅ N ₃ O ₄	PP	2.81	0.0123
211.1488	7.45	Pro Ile	C ₁₁ H ₂₀ N ₂ O ₃	PP	4.16	0.0227
256.1775	4.93	Val Arg	C ₁₁ H ₂₃ N ₅ O ₃	PP	3.28	0.0355
566.317	7.45	Phe Phe Pro Arg	C ₂₉ H ₃₉ N ₇ O ₅	PP	3.06	0.0461

¹AA = amino acid metabolites; PP = small peptides; C/E = carbohydrate and energy metabolites; LL = lipid metabolites; Vit = vitamins and related metabolites; SM = biosynthesis of secondary metabolites; 0 = unknown pathway.

²PC = phosphatidylcholine.

carboxylesterases (Thomas, 2015). Besides, the level of phosphocholine, the structural component of mammalian membrane, also decreased in the milk samples from clinical mastitis cows, which is in agreement with the observation of Thomas (2015). Carnitine metabolism is another metabolic pathway that was downregulated during mastitis. Carnitine is responsible for the transportation of long-chain fatty acids from the cytosol to the mitochondrial matrix and in turn further regulates the energy metabolism (Sundekilde et al., 2011). Acylcarnitine plays an important role in β -oxidation of free fatty acid (Zhou et al., 2012). With the vital functions of carnitine and acylcarnitine as mediators in shut-

tling long-chain fatty acid into the mitochondria for β -oxidation, the decreases in L-carnitine in both the subclinical and clinical mastitic milk samples may suggest a hampered energy metabolism in bovine mastitis.

We observed an increased abundance in small peptides (less than or equal to 4 AA residues in length) in milk samples from clinical and subclinical mastitis cows. Mansor (2012) also reported an elevated level of di- and tri-peptides in mastitic milk samples. Moreover, more milk-derived small peptides were identified in the clinical mastitis cows than in the subclinical group. This is in line with the observation of Thomas et al. (2016) that the metabolism of di-, tri-, and tetra-pep-

Table 7. Significantly differing metabolites between milk samples from subclinical and clinical mastitis cows, negative ion mode analysis

Row <i>m/z</i>	Retention time (min)	Identity of differential metabolites	Molecular formula	Metabolite type ¹	Fold change	<i>P</i> -value
Reduced in clinical mastitis group						
451.379	3.88	5-Tetradecenoic acid	C ₁₄ H ₂₆ O ₂	LL	5.04	1.63 × 10 ⁻¹⁴
677.4259	4.82	PA [15:1(9Z)/20:5(5Z,8Z,11Z,14Z,17Z)] ²	C ₃₈ H ₆₃ O ₈ P	LL	2.76	1.78 × 10 ⁻¹⁴
Increased in clinical mastitis group						
247.1259	12.92	Leu Ala	C ₉ H ₁₈ N ₂ O ₃	PP	172	1.10 × 10 ⁻¹⁶
409.3068	11.99	<i>N</i> -oleoyl glutamine	C ₂₃ H ₄₂ N ₂ O ₄	0	2.88	0.00013
480.3476	11.97	PC (O-16:0/0:0) ³	C ₂₄ H ₅₂ NO ₆ P	LL	6.99	0.00028
435.31	11.97	Ile Ile Ile Pro	C ₂₃ H ₄₂ N ₄ O ₅	PP	5.46	0.00042
494.3556	11.97	Leu Arg Leu Ile	C ₂₄ H ₄₇ N ₇ O ₅	PP	4.57	0.00056

¹PP = small peptides; LL = lipid metabolites; 0 = unknown pathway.

²PA = phosphatidic acid.

³PC = phosphatidylcholine.

tides gradually increased over the time course postchallenge. The increase in small peptides (e.g., Leucyl-Leu, Lys-Asp-Tyr, and Ile-Val-Val in the subclinical mastitic milk samples; Leu-Leu, Leu-Pro-Ile, Arg-Thr-Lys-Arg, and others in the clinical mastitic milk samples) may be due to the activities of proteolytic enzymes of endogenous or bacterial origin, or both (Moussaoui et al., 2002; Wedholm et al., 2008; Larsen et al., 2010). Proteases have different milk protein cleavage specificities, giving rise to a variety of small peptides detected in the mastitic milk samples (Napoli et al., 2007; Larsen et al., 2010). On the other hand, it has also been proposed that small peptides are associated with the milk somatic cells, as the milk SCC correlates with both the protease release (and subsequent proteolysis activity) and the severity of mastitis (Wedholm et al., 2008). Moreover, more free AA (e.g., arginine, valine, isoleucine, and proline) were found in the subclinical and clinical mastitic milk samples, as seen in Mansor (2012). Sundekilde et al. (2013b) also reported the correlation between the milk isoleucine and SCC levels. The increase in milk free AA may be linked with higher pathogen-specific fermentative and protein degradation activities. Thus, the milk AA and peptide profiles may serve as interesting metabolite biomarkers for bovine mastitis diagnosis; however, further experimental validation will be necessary.

Additionally, the levels of hippurate and benzoic acid, the metabolites of phenylalanine metabolism, decreased in the clinical and subclinical mastitic milk samples, respectively. The amount of milk hippurate

was significantly reduced during the course of mastitis induced by *Streptococcus agalactiae* inoculation (36 h postinfections; Thomas, 2015). Benzoic acid is a precursor of hippurate that can inhibit coliform growth (Knarreborg et al., 2002). Therefore, a higher amount of benzoic acid present in the healthy milk samples may protect the mammary gland from *Escherichia coli* invasion. Besides, the milk tyrosine metabolism was also altered in clinical mastitis. 4-Hydroxyphenyllactate is a tyrosine analog, which may be produced in considerable amount by the genera *Bifidobacterium* and *Lactobacillus*. The decrease in milk 4-hydroxyphenyllactate in clinical mastitis cows may be explained by the diminishing of milk *Lactobacillus* as found in a microbial profile analysis of bovine mastitis (Ma et al., 2016). This compound serves as a natural antioxidant that suppresses reactive oxygen species production in both the mitochondria and neutrophils (Beloborodova et al., 2012).

The current untargeted metabolomics approach has revealed numerous significant differences between the milk metabolome of healthy, subclinical, and clinical mastitis cows. There is a decreasing trend in carbohydrate, energy, and lipid metabolism in clinical mastitis cows. Meanwhile, both subclinical and clinical mastitis states were accompanied with an upregulation of AA metabolism, as well as increases in the abundance of short peptides (2 to 4 AA). Apart from confirming some known bovine mastitis biomarkers (e.g., glucose, L-carnitine, isoleucine, hippurate, and sn-glycero-3-phosphocholine), we identified new metabolites such

Table 8. Metabolic pathway analysis of differential metabolites of subclinical and clinical mastitis cow milk

Super pathway	Sub pathway	Metabolites	Response of subclinical mastitis group ^a	Response of clinical mastitis group
Carbohydrate and energy metabolism	Galactose metabolism	Glucose	NSD	↓ ^b
		Lactose	NSD	↓ ^c
		Glucose-1-phosphate	NSD	↓ ^c
	Krebs circle	Citrate	NSD	↓ ^b
		(S)-Malate	NSD	↓ ^b
		cis-Aconitate	↓	↓ ^c
		Oxoglutarate	NSD	↓ ^c
Lipid metabolism	Glycerophospholipid metabolism	Phosphocholine	NSD	↓ ^b
		sn-glycero-3-Phosphocholine	NSD	↓ ^b
		Glycerol-1-phosphate	↓	↓ ^b
	Carnitine metabolism	L-Carnitine	↓	↓ ^b
		O-propanoyl-carnitine	↓	NSD
Protein metabolism	AA/peptides	Small peptides	↑	↑ ^b
		Val, Ile, Pro, Arg	↑	↑ ^b
		4-Hydroxyphenyllactate	NSD	↓ ^{b,c}
	Tyrosine metabolism	4-Hydroxyphenylpyruvate	NSD	↓ ^b
		Hippurate	NSD	↓ ^b
		Benzoic acid	↓	NSD

^a and ^b are the comparison between the respective mastitis group and the healthy one. ^c is the comparison between the clinical mastitis group and the subclinical mastitis one. NSD = no significant difference between the respective mastitis group and the healthy group or subclinical mastitis group.

as D-glycerol-1-phosphate, 4-hydroxyphenyllactate, and benzoic acid that varied greatly between the healthy and mastitic groups. Moreover, 5-methoxytryptophan and indolylacryloylglycine, which decreased in both the subclinical and clinical mastitis groups, are possible markers that deserve further exploration. The present results would help in better understanding the pathobiology of mastitis and mastitis diagnosis.

However, one limitation of this study was the relatively small sample size ($n = 20$ per group). Further experimental validations will be necessary before the current findings can be applied in the farm condition. Moreover, our previous study has observed drastic differences in the milk microbial compositions between healthy and mastitic milk samples (Ma et al., 2016). The microbial composition is anticipated to have a significant influence on the milk metabolome. Thus, genomics analyses will be useful in understanding the interaction between the milk metabolites and milk microbes. Specific proteomics, peptidomics, and lipidomics can also be applied to analyze the protein, peptide, and lipid compounds. With the integrated omics approaches, the pathological and biologic responses to mastitis could be further elucidated.

CONCLUSIONS

By using UPLC-Q-TOF-MS^E technology to comparatively analyze the milk metabolome of samples collected from healthy, subclinical, and clinical mastitis cows, numerous differential metabolites, including glucose, citrate, hippurate, L-carnitine, D-glycerol-1-phosphate, 4-hydroxy-phenyllactate, sn-glycero-3-phosphocholine, benzoic acid, valine, isoleucine, arginine, proline, and Leu-Leu were identified, and it merits further experiments to investigate the potential of using these differential metabolites as biomarkers for mastitis diagnosis, particularly for defining the disease staging. Moreover, the present work shows that the use of UPLC-Q-TOF-MS^E technology in milk metabolome analysis is a promising approach in reliable diagnosis of bovine mastitis.

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