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Milk metabolomic alterations correlated with intramammary infection in dairy cows: From healthy status to clinical mastitis

A. Lisuzzo,[1*] M. C. Alterisio,[2*] S. Esposito,[2] L. Laghi,[3] A. Pesce,[4] P. Ciaramella,[2] F. Cecchini,[1] G. Taio,[1] M. Berlanda,[1] M. Gianesella,[1] J. Guccione,[2†] and E. Fiore[1]
[1]Department of Animal Medicine, Production and Health, University of Padua, 35020 Legnaro, Italy
[2]Department of Veterinary Medicine and Animal Productions, University of Napoli Federico II, 80137 Napoli, Italy
[3]Department of Agricultural and Food Science, University of Bologna, 47521 Cesena, Italy
[4]Istituto Zooprofilattico Sperimentale del Mezzogiorno, 81100 Caserta District, Italy

ABSTRACT

Mastitis is an udder inflammation and infection causing several economic losses in dairy cows. The milk metabolomic changes associated with clinical or subclinical mastitis have been investigated. However, little is known on milk metabolome associated with intramammary infection. The aim of this study was to investigate the quarter-milk metabolomic profile affected by intramammary infection, subclinical mastitis, and clinical mastitis in dairy cows. A total of 80 quarter-milk samples of multiparous Holstein-Friesian dairy cows were used in this cross-sectional design study. Samples were equally divided into 4 groups: healthy (H; no clinical signs of mastitis, SCC <200,000 cells/mL, negative at bacteriological analysis); intramammary infection (IMI group; no clinical signs of mastitis, SCC <200,000 cells/mL, positive at bacteriological analysis); subclinical mastitis (SCM; no clinical signs of mastitis, SCC ≥200,000 cells/mL, positive at bacteriological analysis); and clinical mastitis (CM; clinical signs of mastitis, SCC ≥200,000 cells/mL, positive at bacteriological analysis). Statistical analysis was conducted by fitting a linear mixed model with the group as the fixed effect, quarter nested within animal as random effect, and DIM as covariate. Analysis identified 45 metabolites, and among them 34 were significantly different among groups. Among these, 18 metabolites (2-aminoadipate, Ala, creatine-phosphate, dimethylamine, N-acetyl-Gly, O-phosphocholine, glucose, lactose, maltose, *cis*-aconitate, carnitine, fumarate, lactate, phenylacetate, 2-ketobutyrate, acetoacetate, citicoline, and orotate) progressively changed from the H to the CM stage, and other 12 metabolites (Leu, taurine, Val, arabinose, galactose, ribose, acetate, formate, pyruvate, 5-dodecenoic acid, 3-hydroxybutyrate, and ascorbate) differed only in the CM group. These metabolites were related to blood-milk barrier damage, inflammation, oxidative stress, cell proliferation, energy and lipid metabolisms, the citrate (TCA) cycle, systemic energy status, and microorganism metabolism. These results suggest that metabolomic alterations in milk begin to occur when the mammary gland is infected with somatic cells within normal limits and progressively worsen.
Key words: bovine mastitis, intramammary infection, metabolomics, udder health, milk metabolites

INTRODUCTION

Mastitis is an inflammation and infection of the mammary gland caused by various types of microorganisms, usually bacteria, but also yeasts, algae, and viruses (Antanaitis et al., 2021; Haxhiaj et al., 2022). This disease is considered one of the main concerns in dairy cow farming, as it negatively affects animal health and welfare, milk production and quality, treatment costs, risk of excessive antibiotic use, and cow mortality (Guccione et al., 2024). Furthermore, mastitis can affect between 25% and 60% of a dairy herd (Zhu et al., 2021; Du et al., 2024).

The main route of udder infection is the teat duct. When the pathogen crosses this barrier and infects the mammary tissue, it triggers innate immune and inflammatory responses characterized by neutrophils, macrophages, natural killer cells, dendritic cells, mammary epithelial cells, and humoral defenses. These immune and inflammatory responses are responsible for the increase in SCC in milk. In addition, increased immune cell activity leads to increased oxidative stress due to reactive oxygen species and the release of antimicrobial peptides. If the pathogen is eliminated, the SCC gradually returns to a healthy homeostatic state (Haxhiaj et al., 2022). For this reason, SCC are usually used to diagnose mastitis (John-

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*These authors contributed equally to this work.
†Corresponding author: jacopo.guccione@unina.it

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zon et al., 2018; Du et al., 2024). A threshold of 200,000 cells/mL is usually indicative of infection and inflammation of the mammary gland tissue (Dervishi et al., 2017; Xi et al., 2017; Haxhiaj et al., 2022). Nevertheless, the gold standard to diagnose an udder infection is bacteriological analysis of milk, even if this method has the disadvantage of being time-consuming (Du et al., 2024). In addition, some intramammary infections are not associated with increased milk SCC (Pegolo et al., 2022).

Mastitis can be classified as clinical or subclinical based on the presence or absence, respectively, of clinical signs with an increase in SCC (Antanaitis et al., 2021). The duration and severity of clinical signs, respectively, are used to classify clinical mastitis as subacute, acute, peracute, or chronic, and as mild, moderate, or severe (Haxhiaj et al., 2022; Li et al., 2024). Mild mastitis is characterized by changes only in milk color and consistency with appearance of clots, whereas moderate is characterized by changes in the milk and mammary gland, including redness, swelling, heat, and pain (Johnzon et al., 2018; Haxhiaj et al., 2022). Severe mastitis also has systemic signs such as fever, loss of appetite, and reduced sensory state (Haxhiaj et al., 2022). In all these cases, milk SCC and metabolites are altered (Xi et al., 2017). Metabolites are small molecules present in biological fluids, which are evaluated using different instruments such as nuclear magnetic resonance (**NMR**) spectroscopy (Luangwilai et al., 2021). The study of metabolites is called metabolomics, and the set of metabolites in a biofluid is called the metabolome or metabolomic profile (Zhu et al., 2021). This analysis is considered the most comprehensive representation of the physiological or pathological state of an organism. However, the metabolomics investigation must take into account the evaluated biofluid (Du et al., 2024; Lisuzzo et al., 2024). In fact, the milk metabolome can be influenced by systemic conditions, secretion or leakage by inflammatory, immune, and mammary epithelial cells, and bacterial metabolism (Zhu et al., 2021). Several studies have been conducted to study milk metabolomic profiles. However, knowledge about the mechanisms by which milk metabolites are altered, and the influence of intramammary infection by itself on the milk metabolome is still lacking (Luangwilai et al., 2021; Du et al., 2024).

In this study, we hypothesized that intramammary infection, subclinical mastitis, and clinical mastitis were associated with different changes in the milk metabolome that could be useful for better understanding how mastitis can affect the milk metabolomic profile. To test this hypothesis, the study investigated the quarter-milk metabolomic profile of dairy cows affected by these conditions.

MATERIALS AND METHODS

Ethical Statement

Animal care and procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and Directive 2010/63/EU for animal experiments (European Union, 2010). All clinical procedures performed during the routine mastitis monitoring program complied with common good clinical practices (European Medicines Agency, 2025) and received formal ethical approval from the Ethics Committee for the Care and Use of Animals of the University of Naples Federico II (PG/2024/0023594) because they were conducted as part of a Research Project of National Interest (call 2022, protocol number 2022YME8Y7). Furthermore, the farmer was informed in advance, and written consent was also received.

Animals and Study Design

The study was conducted in a freestall barn located in Campania (Italy) with 1,800 lactating Holstein-Friesian dairy cows between March and November 2024. The dry period began about 60 d from the expected calving with a steaming-up during the last 3 wk of pregnancy. Cows were fed a TMR twice daily, and water was provided ad libitum for all animals. In addition, the farm applied a regular mastitis monitoring program, including regular SCC and veterinary clinical examinations.

The study used a cross-sectional design. Quarters were enrolled respecting the following inclusion criteria. (1) Twenty quarters for each udder health status grouping: clinical mastitis (**CM**), subclinical mastitis (**SCM**), intramammary infection (IMI), and healthy (**H**); (2) belonging to multiparous cows (≥second lactation); (3) between 30 and 100 DIM; (4) not under antimicrobial treatment and without history of treatment in the previous 6 mo; and (5) without any other disease (i.e., retained placenta, metritis, pneumonia, ketosis, displaced abomasum, lameness). For this reason, each animal underwent a thorough clinical examination as described by Jackson and Cockcroft (2002), with particular attention to the teat and udder (Guccione et al., 2017; Alterisio et al., 2021). Finally, (6) all cows received a California Mastitis Test (**CMT**) in the milking parlor, and, to minimize the influence of any concomitant mammary pathological conditions, the enrolled cows were required to have only 1 quarter affected by mastitis (clinical or subclinical) with CMT score ≥1, whereas those affected by intramammary infection and healthy cows were required to have CMT = 0 on all 4 quarters.

Milk sampling was performed at the end of the examination procedures, following National Mastitis Council guidelines (NMC, 2017). The teat ends were cleaned with commercial pre-milking disinfectants, dried with individual towels, and then cleaned again with 70% alcohol. The first streams of foremilk were then discarded. Immediately after, ~60 mL of milk was manually collected from each quarter in a single sterile tube (Falcon, Thermo Fisher Scientific, Segrate, Italy) and subsequently divided into 2 aliquots in separate test tubes (30 mL in each; Falcon, Thermo Fisher Scientific, Segrate, Italy), and stored at 4°C for further investigation. One sample was transferred to laboratory for SCC (Fossomatic 5000 system, Foss Electric, Hillerod, Denmark) and bacteriological milk cultures (**BMC**) within 1 h; the other was stored at −80°C until metabolomic investigations were carried out.

Milk Microbiological Analysis

Laboratory procedures were performed following the diagnostic procedures adopted in previous studies (Guccione et al., 2020). Briefly, BMC and bacteriological identification were performed following the guidelines of the National Mastitis Council (NMC, 2017). Both tests were blindly performed by the same investigator in duplicate to confirm the BMC results and correctly assign bacteriological positivity. In detail, 10 μL of each milk sample was smeared on one-quarter of a blood agar plate (Merck KGaA, Darmstadt, Germany), incubated at 37°C for up to 48 h, and analyzed twice (at 24 and 48 h after incubation). Bacterial colonies were initially identified based on macroscopic morphology. The number and types of colonies present were also recorded. As described previously (Guccione et al., 2020), when 3 or more different types of colonies were isolated on the plate, the sample was considered contaminated. To distinguish between streptococci and staphylococci spp., Gram staining and catalase tests were performed, and the coagulase test in a test tube with rabbit plasma was used to differentiate between coagulase-positive and coagulase-negative staphylococci. The final identification of microorganisms was performed using an automated colorimetric system (Vitek 2 XL 120; bioMerieux Inc., Hazelwood, MO). *Enterobacteriaceae* were cultured on MacConkey agar (Oxoid, Basingstoke, UK) and identified using the same automated system. Mastitis-causing pathogens were identified at the species level with confidence levels above 0.90; otherwise, they were identified at the genus level. One colony-forming unit per milliliter was considered the threshold for assigning positivity for *Staphylococcus aureus*.

Milk Metabolomic Analysis

Milk samples were prepared for ^{1}H-NMR analysis by centrifuging 2 mL of each sample for 15 min at 18,630 × *g* and 4°C. The liquid below the fat layer was then withdrawn and centrifuged once more under the same conditions. A final aliquot of 0.7 mL was centrifuged one final time with 0.1 mL of an NMR analysis solution. This consisted in a D_2O solution of 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid-sodium salt (**TSP**) 10 mmol/L, set at pH 7.00 ± 0.02 by means of 1 mol/L phosphate buffer containing 2 mmol/LNaN_3. The TSP was used as an NMR chemical shift reference, while NaN_3 avoided microbial proliferation.

The ^{1}H-NMR spectra were recorded at 298 K with an Avance III spectrometer (Bruker, Milan, Italy) operating at a frequency of 600.13 MHz, equipped with the software Topspin 3.5. The signals from broad resonances originating from large molecules were suppressed by a Carr-Purcell-Meiboom-Gill filter comprising 400 echoes with τ of 400 μs and a 180° pulse of 24 μs for a total filter of 330 ms. The water residual signal was suppressed using a pre-saturation technique. This setting employs the cpmgpr1d sequence, part of the standard pulse sequence library. Each spectrum was acquired by summing 256 transients using 32 K data points over a 7,184 Hz spectral window with an acquisition time of 2.28 s and a relaxation delay of 5s. The spectral phase was then manually adjusted in Topspin, and subsequent adjustments were performed in R computational language using a script developed in-house. Signals were attributed to individual metabolites by comparing their chemical shifts, line shapes, and multiplicities with reference data from the Human Metabolome Database (Wishart et al., 2007) and the Chenomx library (Chenomx Inc., Edmonton, AB, Canada; version 10), using the identification routines integrated in the Chenomx software. To enable absolute quantification of metabolite concentrations, one sample was supplemented with a known amount of maleic acid. Spectra were then normalized through the probabilistic quotient normalization method (Dieterle et al., 2006) to correct for possible differences in water content among samples. Following the approach described by Brugaletta et al. (2023), metabolite concentrations were derived from the area of selected resonances, calculated using the global spectral deconvolution (GSD) algorithm implemented in MestReNova software (version 14.2.0-26256; Mestrelab Research S.L., Santiago de Compostela, Spain). A limit of quantification of 5 was applied. Before integration, spectra were baseline-corrected using the Whittaker smoother method and processed with 0.3-Hz line broadening. An example of sample spectra and

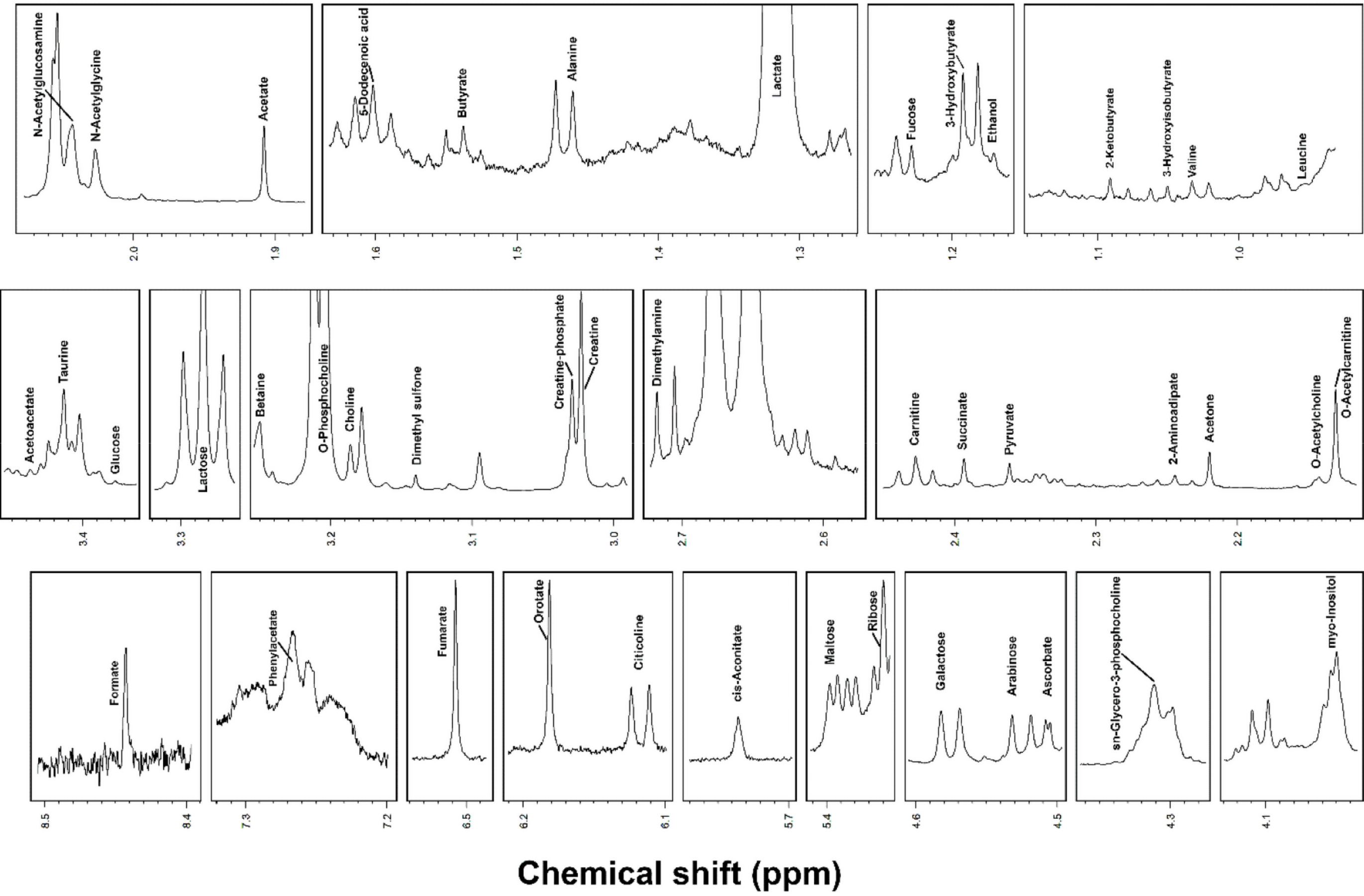

Figure 1. Example of ^{1}H-NMR spectra, representative of all the spectra obtained in this study. Each molecule's name appears over the NMR peak used for its quantification. To ease visual inspection of each portion, a different spectrum with a convenient signal-to-noise ratio has been selected.

lever of identification according to Sumner et al. (2007) are presented in Figure 1 and Supplemental Table S1 (see Notes), respectively.

Udder Health Status

To fulfill the categorization criteria while meeting the previously mentioned eligibility requirements, a total of 250 animals (overall 1,000 quarters) were simultaneously selected and screened by means of CMT. Following bacteriological analysis, quarter-milk samples with contaminated BMC results were excluded. This process achieved the goal of recruiting 20 quarters per category, as previously reported by Alterisio et al. (2021), for a total of 80 quarters enrolled. The enrolled quarters were equally divided into 4 groups as follows: (1) quarters producing milk with SCC $\geq 200 \times 10^3$ cells/mL and positive BMC for recognized mastitis-causing pathogens were classified as CM (in presence of clinical signs) or SCM (in absence of clinical signs); (2) quarters producing milk with SCC $<200 \times 10^3$ cells/mL and positive BMC for recognized mastitis-causing pathogens were classified as IMI; (3) quarters producing milk with SCC $<200 \times 10^3$ cells/mL and a negative BMC were defined as H.

Statistical Analysis

The sample size was calculated according to Friedman (1982) guidelines, considering a correlation analysis with 0.90 power level, a 2-tailed significance level of 0.05, and an assumed effect size of 0.40.

MetaboAnalyst 6.0 software (https://www.metaboanalyst.ca) was used to study the group clustering and the influenced metabolic pathways by milk metabolome. The sparse partial least squares discriminant analysis (**sPLS-DA**) was used as supervised method of clustering technique to assess differences among observations. A hierarchical clustering heatmap was then generated using all identified metabolites and applying autoscaling data (mean-centered and divided by the standard deviation of each variable) to identify metabolome differences among groups. The metabolites' clusters and subclusters, identified by the heatmap, were used for pathway analysis. Pathway analysis was performed us-

Table 1. General characteristics as mean (range in parentheses) in healthy quarters (H; n = 20), intramammary infection quarters (IMI; n = 20), subclinical mastitis quarters (SCM; n = 20), and clinical mastitis quarters (CM; n = 20)

General characteristic	H (n = 20)	IMI (n = 20)	SCM (n = 20)	CM (n = 20)	SEM	*P*-value
Parity	3.15 (2–5)	2.75 (2–4)	2.80 (2–4)	3.55 (2–5)	0.80	0.395
DIM	71.9 (51–93)	71.8 (46–95)	68.8 (46–95)	71.2 (51–89)	13.7	0.880
SCC ($\times 10^3$ cells/mL)	49.2^C (5.5–114.5)	66.1^C (4–120)	$1,982.8^B$ (235–9,364.5)	$11,834.7^A$ (332.5–21,048.5)	304.4	<0.001

[A–C]Different superscripts indicate significant change between groups ($P \leq 0.05$).

ing the *Bos taurus* pathway library (Kyoto Encyclopedia of Genes and Genomes [**KEGG**] pathway information obtained in December of 2024). In this analysis, the software automatically used a Fisher's exact test with *P*-values corrected by the Holm Bonferroni correction to identify significantly influenced pathways.

Statistical analysis was performed with R software v.4.2.3 (https://www.R-project.org/). Normal distribution of data was confirmed using the Shapiro–Wilk test. The Akaike information criterion (**AIC**) and the Bayesian information criterion (**BIC**) were used to select the model based on performance (lowest AIC and BIC). The selected linear mixed model included the fixed effect of group (4 groups: H, IMI, SCM, and CM), udder quarter nested within animal as random effect, and the covariate of DIM. A post-hoc pairwise comparison among LSM was performed using Tukey correction. In addition, a post-hoc power analysis was also performed on the results to ensure a power equal or greater than 0.80. A *P*-value ≤0.05 was used to consider statistically significant differences, and a *P*-value between 0.05 and 0.1 was used to highlight differences with trend to significance.

Using MetaboAnalyst 5.0 software (https://www.metaboanalyst.ca), the PubChem (https://pubchem.ncbi.nlm.nih.gov/), HMDB (https://hmdb.ca/metabolites/), and KEGG websites (https://www.genome.jp/kegg/) were consulted to assess the functions of significant and nonsignificant metabolites (Shi et al., 2021).

RESULTS

General characteristics of the studied cows (parity, DIM, and SCC) are presented in Table 1. Among them, only SCC was significantly different among groups according to group division. Bacteriological analyses of quarters milk samples were all positive for the IMI, SCM, and CM groups and negative for the H group. The IMI group comprised 50% of udder quarters with monoinfection, and 50% with coinfection; the SCM group had 65% with monoinfection and 35% with coinfection; and the CM group had 75% monoinfection and 25% coinfection. Identified pathogens are presented in Table 2. All quarters showing clinical signs of mastitis presented abnormal, watery milk with flakes and clots. Swelling and pain of the udder and teats were also observed in all affected quarters.

A total of 45 metabolites were identified by metabolomic analysis with ^{1}H-NMR spectroscopy and used for chemometric and cluster analysis. The sPLS-DA was used as the supervised method to produce robust and easy-to-interpret models for groups' clustering. The projections of the 3-dimensional plot (Figure 2) showed a clear separation of the CM group versus all other groups. Additionally, the SCM group was separated from the H group, but it presented a partial division from IMI group. Finally, the IMI group was partially separated from the H group, with a greater metabolome distribution. Metabolites used for components 1, 2, and 3 are presented in Supplemental Table S2 (see Notes).

The hierarchical clustering heatmap (Figure 3) showed a separation between CM metabolome versus all other groups. Moreover, the metabolomes of the IMI and SCM groups were also divided by the H group. The metabolites evinced 2 main clusters. The first had 3 subclusters (subclusters 1, 2, and 3), and the second had 2 subclusters (4 and 5). Subcluster 1 included metabolites with lower concentrations in the CM group versus all other groups; subcluster 2 included metabolites with lower concentrations in the CM group versus the IMI and SCM groups; subcluster 3 included metabolites with lower concentrations in all infected groups; subcluster 4 included metabolites with greater concentrations in the CM group versus all other groups; and subcluster 5 included

Table 2. Frequency of identified pathogens in infected quarters

Pathogen	Frequency (%)
Staphylococcus aureus	24.4
Escherichia coli	23.3
Bacillus cereus	9.30
Staphylococcus chromogenes	9.30
Streptococcus uberis	9.30
Staphylococcus lentus	5.81
Staphylococcus lerus	4.65
Coagulase-negative *Staphylococcus*	4.65
Serratia marcescens	4.65
Staphylococcus warneri	3.49
Bacillus spp.	1.16

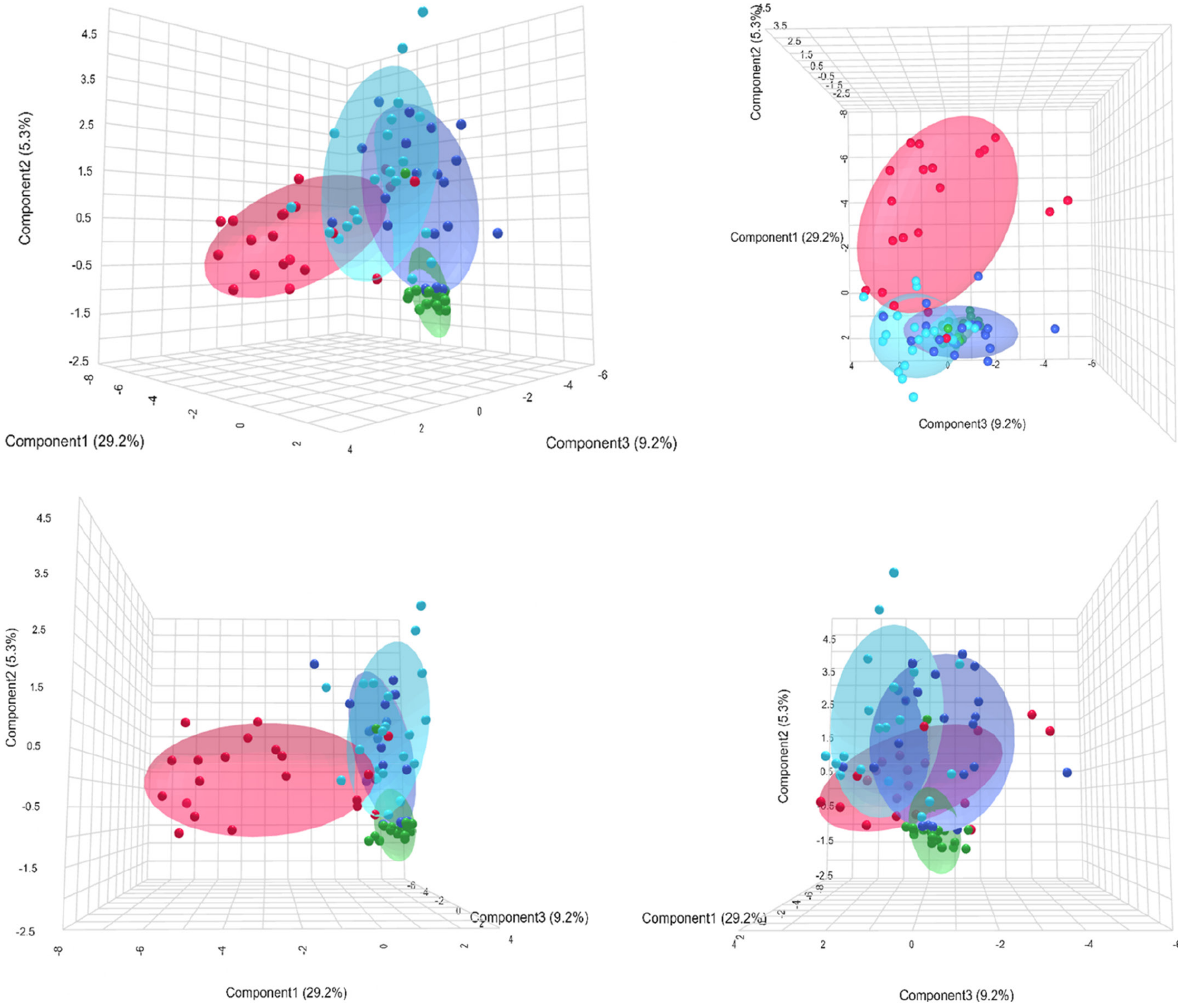

Figure 2. Three-dimensional sparse PLS-DA (sPLS-DA) plots, generated by MetaboAnalyst 6.0 (https://www.metaboanalyst.ca). Green = metabolome distribution of healthy quarters (H; n = 20); blue = metabolome distribution of intramammary infection quarters (IMI; n = 20); light blue = metabolome distribution of subclinical mastitis quarters (SCM; n = 20); and red = metabolome distribution of clinical mastitis quarters (CM; n = 20).

metabolites with greater concentrations in the CM group versus the IMI and SCM groups. All the subclusters were used for pathway analysis. However, only subclusters 1, 2, and 4 had significant results. The influenced pathways were glycine, serine and threonine metabolism (Holm P = 0.005), glycerophospholipid metabolism (Holm P = 0.006), the citrate (**TCA**) cycle; Holm P = 0.019), and galactose metabolism (Holm P = 0.034) for subcluster 1; glycerophospholipid metabolism (Holm P = 0.002) for subcluster 2; and pyruvate metabolism (Holm P = 0.002) and glycolysis or gluconeogenesis (Holm P = 0.003) for subcluster 4 (Figure 4).

A total of 34 of 45 metabolites were significantly different among groups (Table 3): 2-aminoadipate ($P < 0.001$), 2-ketobutyrate (P = 0.017), 3-hydroxybutyrate (P = 0.004), 5-dodecenoic acid (P = 0.026), acetate ($P < 0.001$), acetoacetate (P = 0.006), Ala (P = 0.009), arabinose ($P < 0.001$), ascorbate ($P < 0.001$), betaine (P = 0.046), butyrate (P = 0.039), carnitine ($P < 0.001$), *cis*-aconitate ($P < 0.001$), citicoline ($P < 0.001$), creatine-phosphate (P = 0.008), dimethylamine (P = 0.042), formate (P = 0.011), fumarate (P = 0.016), galactose ($P < 0.001$), glucose (P = 0.042), lactate ($P < 0.001$), lactose ($P < 0.001$), Leu (P = 0.012), maltose (P = 0.020), N-acetylglucosamine (P =

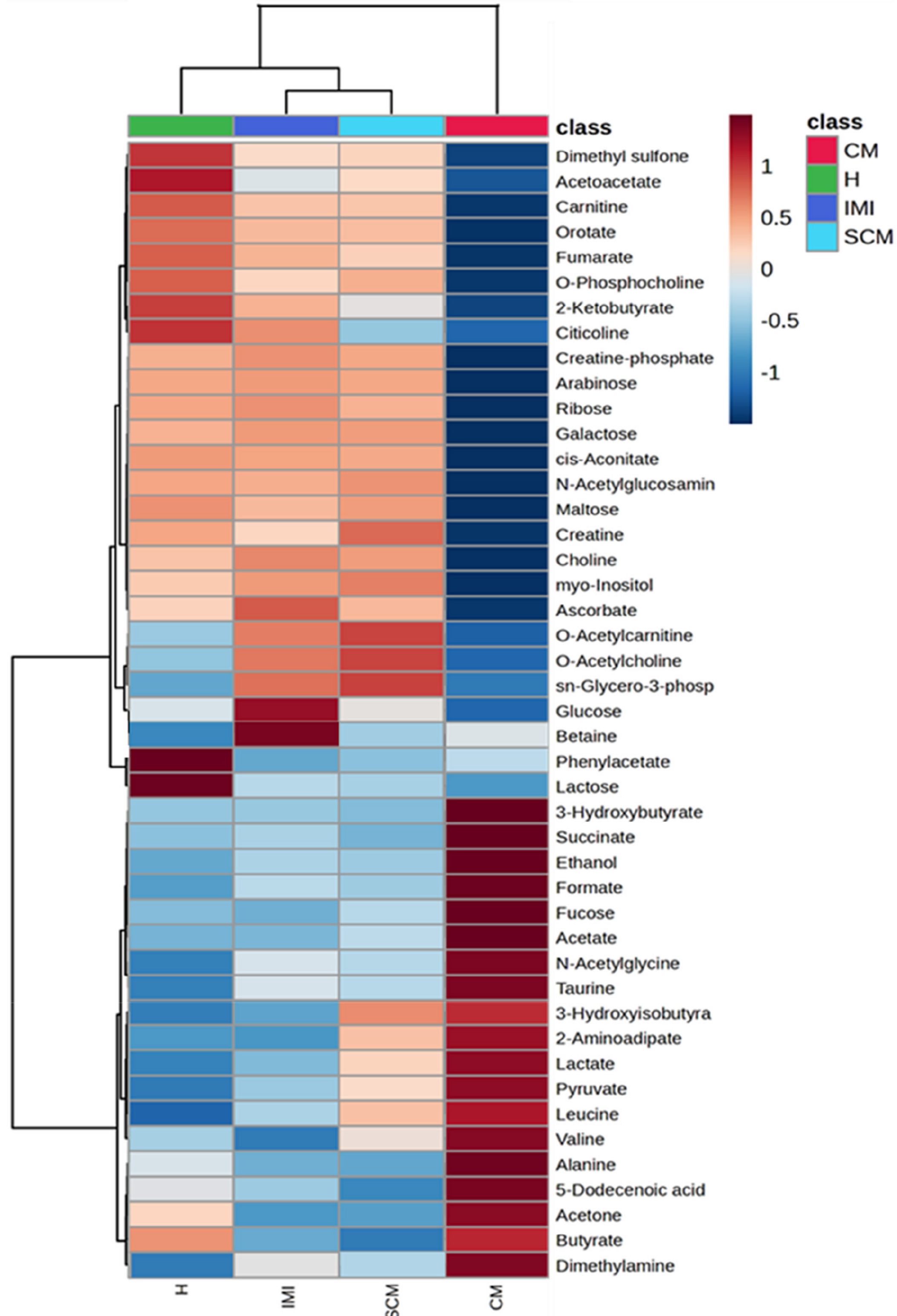

Figure 3. Hierarchical clustering heatmap of all identified metabolites as group average, generated by MetaboAnalyst 6.0 (https://www.metaboanalyst.ca). On the top right is the colorimetric scale: if the color tends to dark red, then the metabolite's concentration was increased; if the color tends to dark blue, then the metabolite's concentration was decreased. The green, blue, light blue, and red boxes on the upper right correspond, respectively, to healthy quarters (H; n = 20), intramammary infection quarters (IMI; n = 20), subclinical mastitis quarters (SCM; n = 20), and clinical mastitis quarters (CM; n = 20) and are represented in the uppermost row of the heatmap. On the left side of the heatmap is the clustering division.

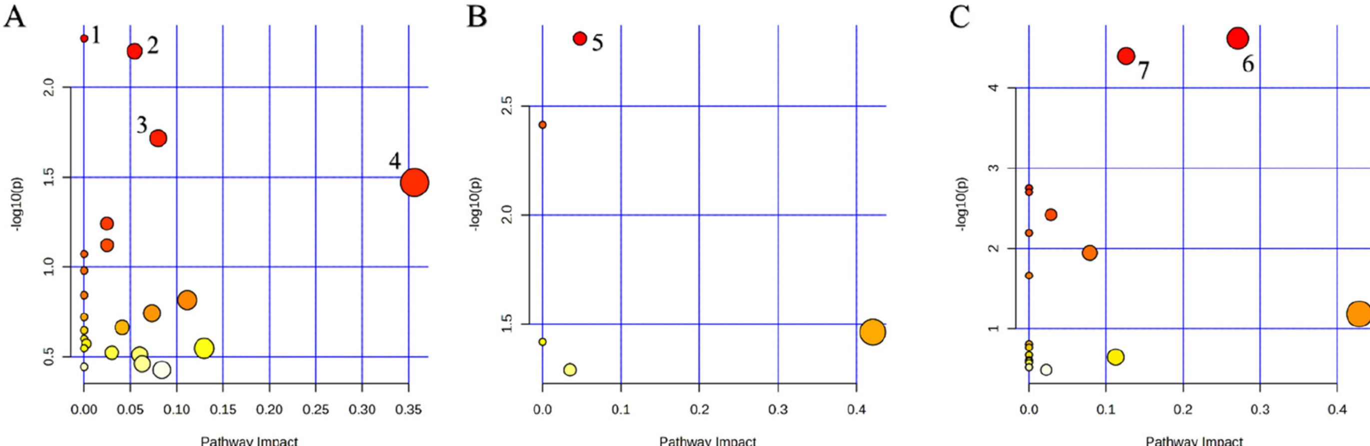

Figure 4. Bubble plot of significant metabolic pathways influenced by (A) subcluster 1 (lower concentration in the clinical mastitis group versus all other groups), (B) subcluster 2 (lower concentration in the clinical mastitis group vs. intramammary infection and subclinical groups), and (C) subcluster 4 (greater concentration in the clinical mastitis group than all other groups), generated by MetaboAnalyst 6.0 (https://www.metaboanalyst.ca). Numbering on the bubbles is as follows: (1) glycine, serine, and threonine metabolism; (2) glycerophospholipid metabolism; (3) citrate cycle (TCA cycle); (4) galactose metabolism; (5) glycerophospholipid metabolism; (6) pyruvate metabolism; and (7) glycolysis or gluconeogenesis. Color gradient and symbol size represent significant metabolite changes in the corresponding pathway ($P \leq 0.05$).

0.010), N-acetyl-Gly ($P = 0.002$), O-phosphocholine ($P = 0.033$), orotate ($P < 0.001$), phenylacetate ($P < 0.001$), pyruvate ($P < 0.001$), ribose ($P < 0.001$), sn-glycero-3-phosphocholine ($P < 0.001$), taurine ($P = 0.003$), and Val ($P < 0.001$). Metabolites with changes from H to CM are also presented in Figure 5. A diagram of mastitis metabolic changes is presented in Figure 6. All statistical differences among metabolite concentrations had a power of at least 80% at post hoc analysis. The covariate DIM was associated only with choline, with a negative relationship ($P = 0.012$), and with orotate, with a positive relationship ($P = 0.047$).

DISCUSSION

Inflammatory Condition

Milk is a natural fluid consisting of nutrients such as lactose, protein, lipids, vitamins, minerals, and some metabolites (Sen et al., 2021). Damage to the mammary cell membrane during mastitis leads to blood constituents flowing into the milk. To keep the osmotic pressure constant, milk lactose is decreased accordingly (Zhu et al., 2021). Consequently, the lactose concentration decreases in milk during mastitis and instead increases in blood (Sen et al., 2021; Lisuzzo et al., 2024). This finding was also observed in this study, with a progressive decrease of milk lactose from H to CM cows, even below the reference range (118–160 mmol/L) for lactose in NMR (Klein et al., 2010), suggesting damage to the blood-milk barrier due to inflammation. This hypothesis was further suggested by other metabolites as N-acetyl-glucosamine and 5-dodecenoic acid. N-acetyl-glucosaminidase (**NAGase**) is a lysosomal enzyme released from damaged epithelial cells in the mammary gland, as well as from other somatic cells present in milk. Activity of NAGase has been used as an indicator of the degree of udder inflammation in cows due to its increase during clinical mastitis (Chagunda et al., 2006). Prior studies have found that NAGase hydrolyzes the glycosidic linkage of N-acetyl-glucosamines (Frohwein and Gatt, 1967). In our study, N-acetyl-glucosamine reduced in CM compared with IMI and SCM, probably related to a possible greater amount of NAGase associated with the highest SCC level. However, no significant difference was evidenced between infected udders (IMI, SCM, and CM groups) compared with the H group, in contrast to (Zhu et al., 2021). The enzyme activity has also been related to parity and DIM (Chagunda et al., 2006), and the metabolite has also been found to be related to energy balance (Xu et al., 2020). Consequently, animal characteristics could have led to the lack of differences versus H quarters. However, a greater inflammatory condition could be suspected in milk of CM quarters. In fact, this group had greater levels of 5-dodecenoic acid compared with all other groups. 5-Dodecenoic acid derives from lipid oxidation, and it has moderate activity to inhibit cyclooxygenases, probably contributing to management of inflammatory conditions (Henry et al., 2002; M. T. Al Bataineh [Yarmouk University, Irbid, Jordan], A. S. Barreiros, J. Farhat [Khalifa University of Science and Technology, Abu Dhabi, United Arab Emirates], A. Mayyas [American University of Madaba, Amman, Jordan], N. AlKhayyal [University Hospital of Sharjah, Sharjah,

Table 3. Mean concentrations of all identified milk metabolites (μmol/L) in healthy quarters (H; n = 20), intramammary infection quarters (IMI; n = 20), subclinical mastitis quarters (SCM; n = 20), and clinical mastitis quarters (CM; n = 20)

Class	Metabolite (μmol/L)	H (n = 20)	IMI (n = 20)	SCM (n = 20)	CM (n = 20)	SEM	*P*-value	
							Group	DIM
Alcohols and polyols	Ethanol	122.6	78.4	85.2	190.0	47.2	0.304	0.109
	Myo-inositol	1,717	1,878	1,922	1,626	198.8	0.229	0.427
Amino acids, peptides, amines, and analogs	2-Aminoadipate	36.0^{C}	61.4^{BC}	84.6^{B}	161.6^{A}	15.2	<0.001	0.722
	Alanine	53.0^{B}	51.3^{B}	56.1^{AB}	77.5^{A}	9.63	0.009	0.754
	Betaine	113^{B}	143^{A}	130^{AB}	129^{AB}	12.3	0.046	0.294
	Creatine	675	674	696	569	57.2	0.120	0.478
	Creatine-phosphate	143.4^{A}	167.4^{A}	120.6^{AB}	76.7^{B}	31.2	0.008	0.954
	Dimethylamine	3.28^{B}	4.49^{AB}	3.77^{AB}	5.05^{A}	0.60	0.042	0.779
	Leucine	7.11^{B}	7.66^{B}	9.19^{B}	79.2^{A}	7.90	0.012	0.966
	N-Acetyl glycine	204^{C}	305^{B}	292^{B}	426^{A}	38.6	0.002	0.464
	O-Phosphocholine	245^{A}	230^{A}	167^{B}	173^{B}	28.2	0.033	0.490
	Taurine	208^{B}	275^{B}	264^{B}	469^{A}	55.0	0.003	0.748
	Valine	21.1^{B}	18.0^{B}	35.5^{B}	126.6^{A}	11.6	<0.001	0.782
Carbohydrates and carbohydrate conjugates	Arabinose	534^{A}	554^{A}	536^{A}	257^{B}	33.8	<.001	0.686
	Fucose	44.0	47.6	45.7	56.4	12.2	0.392	0.159
	Galactose	951^{A}	$1,062^{A}$	$1,093^{A}$	646^{B}	83.6	<0.001	0.706
	Glucose	777^{AB}	859^{A}	693^{BC}	559^{C}	67.6	0.042	0.353
	Lactose ($\times 10^3$)	154.1^{A}	145.9^{AB}	139.2^{B}	100.8^{C}	7.66	<0.001	0.372
	Maltose	107.1^{A}	97.8^{AB}	98.3^{AB}	68.4^{B}	14.9	0.020	0.733
	N-Acetylglucosamine	$1,073^{AB}$	$1,216^{A}$	$1,330^{A}$	874^{B}	189	0.010	0.963
	Ribose	365^{A}	400^{A}	342^{A}	211^{B}	34.2	<0.001	0.507
Carboxylic acids, hydroxy acids, and derivatives	*cis*-Aconitate	30.2^{A}	34.8^{A}	27.0^{AB}	18.3^{B}	4.18	<0.001	0.230
	3-Hydroxyisobutyrate	11.0	10.2	14.1	19.0	6.68	0.249	0.803
	Acetate	53.2^{B}	52.5^{B}	100.0^{B}	281.4^{A}	36.6	<0.001	0.347
	Carnitine	280^{A}	266^{A}	228^{B}	123^{C}	15.4	<0.001	0.127
	Formate	15.0^{B}	21.6^{B}	20.7^{B}	59.8^{A}	11.1	0.011	0.467
	Fumarate	38.7^{A}	39.0^{A}	34.2^{B}	31.1^{B}	2.44	0.016	0.418
	Lactate	244^{C}	959^{B}	$1,119^{B}$	$1,951^{A}$	127.8	<0.001	0.947
	O-Acetylcholine	13.7	15.0	16.0	15.4	1.86	0.805	0.985
	Phenylacetate	236.3^{A}	87.2^{B}	101.9^{B}	123.5^{B}	21.1	<0.001	0.748
	Pyruvate	25.2^{B}	29.9^{B}	43.5^{B}	71.3^{A}	9.54	<0.001	0.961
	Succinate	25.6	25.8	24.9	32.0	3.50	0.356	0.493
Fatty acids, esters, and conjugates	5-Dodecenoic acid	137.8^{B}	122.2^{B}	99.2^{B}	345.8^{A}	66.4	0.026	0.361
	Butyrate	29.9^{A}	27.6^{AB}	22.9^{B}	31.2^{A}	2.63	0.039	0.357
	O-Acetyl carnitine	76.8	98.7	95.3	81.3	9.14	0.246	0.919
Glycerophosphocholine	sn-Glycero-3-phosphocholine	$1,448^{B}$	$2,144^{A}$	$1,977^{A}$	$1,256^{B}$	167.3	<0.001	0.187
Ketone bodies, keto acids, and derivatives	2-Ketobutyrate	6.10^{A}	5.59^{AB}	3.92^{BC}	3.24^{C}	0.80	0.017	0.289
	3-Hydroxybutyrate	73.9^{B}	112.6^{B}	90.3^{B}	514.6^{A}	24.0	0.004	0.923
	Acetoacetate	48.6^{A}	38.6^{BC}	43.0^{AB}	30.6^{C}	3.76	0.006	0.810
	Acetone	23.9	22.4	22.4	24.9	2.45	0.952	0.787
Pterins and derivatives	Citicoline	40.3^{A}	24.0^{B}	14.9^{C}	15.1^{C}	4.28	<0.001	0.199
Pyrimidines and pyrimidine derivatives	Orotate	201^{A}	137^{B}	115^{B}	101^{B}	24.8	<0.001	0.047
Sulfones	Dimethyl-sulfone	65.5	59.3	59.9	56.8	5.39	0.688	0.564
Vitamins	Ascorbate	158.9^{A}	157.7^{A}	151.3^{A}	86.8^{B}	16.6	<0.001	0.703
	Choline	694	691	698	612	114.8	0.848	0.012

$^{A–C}$Different superscripts indicate significant change between groups ($P \leq 0.05$).

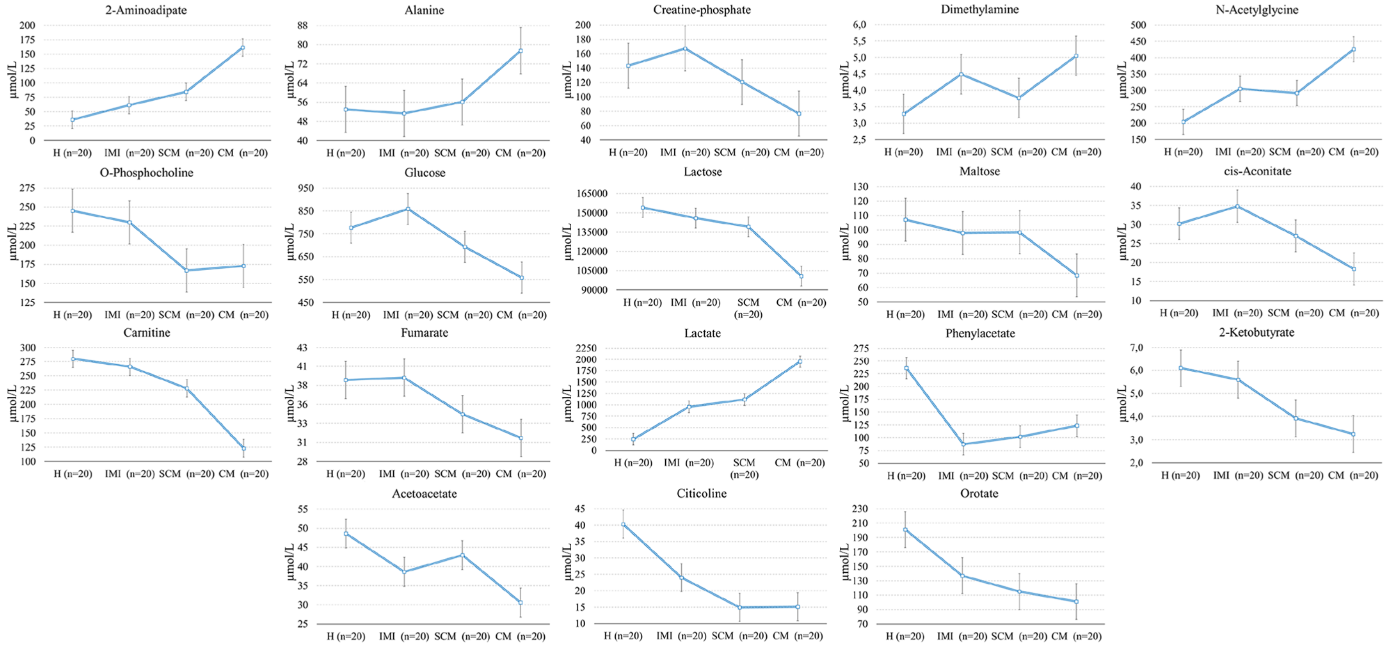

Figure 5. Significant ($P \leq 0.05$) metabolites (μmol/L) with a change from healthy to clinical mastitis quarters. Exact means and differences are presented in Table 2. Quarters were classified as healthy (H), intramammary infection (IMI), subclinical mastitis (SCM), and clinical mastitis (CM). Error bars represent SE.

United Arab Emirates], B. Al-Omari [Khalifa University of Science and Technology], unpublished data). In fact, its concentration was increased in human uncontrolled diabetes and in rats with inflammatory bowel disease (Liu et al., 2019; M. T. Al Bataineh [Yarmouk University, Irbid, Jordan], A. S. Barreiros, J. Farhat [Khalifa University of Science and Technology, Abu Dhabi, United Arab Emirates], A. Mayyas [American University of Madaba, Amman, Jordan], N. AlKhayyal [University Hospital of Sharjah, Sharjah, United Arab Emirates], B. Al-Omari [Khalifa University of Science and Technology], unpublished data).

Mammary Gland Infection

The inflammatory condition of quarters was related to mammary gland infection in the IMI, SCM, and CM groups, as confirmed by results of bacteriological analysis. Moreover, several metabolites related to infection were identified in milk samples, including orotate, di-

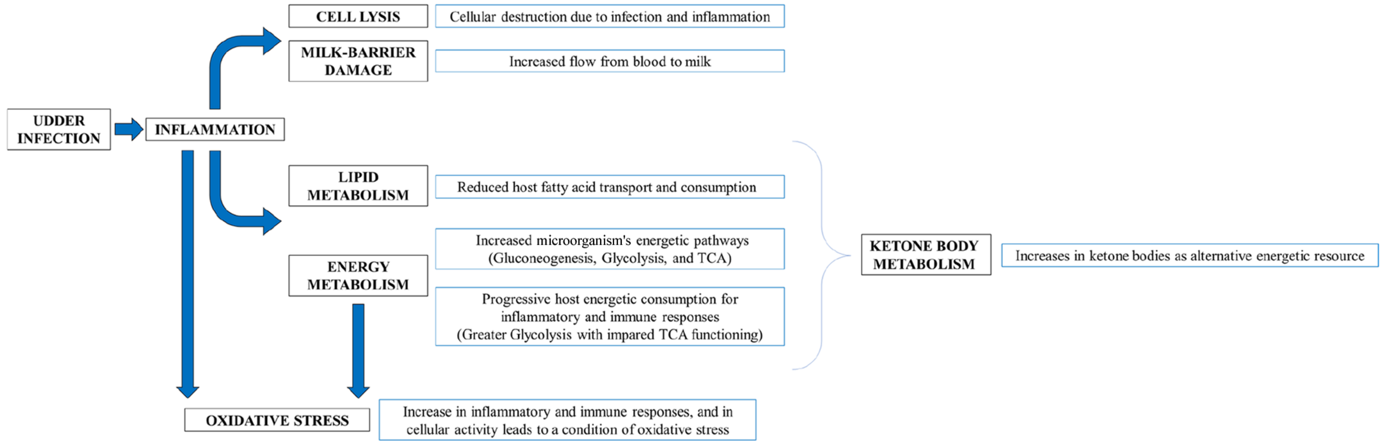

Figure 6. Diagram of mastitis metabolic changes according to metabolic pathways and metabolomic changes.

methylamine, and N-acetyl-Gly. Orotic acid (also known as vitamin B_{13}) is a metabolite characteristic of ruminant milk, with a decreased concentration over lactation up to 30 to 70 μg/mL (Löffler et al., 2015; Ji et al., 2023). This metabolite is produced by the mammary gland for suckling young. However, several bacteria could use orotate as nitrogen source and degrade it to Asp and ammonia (Löffler et al., 2015; Sun et al., 2017). Also linked to the nitrogen pathways of microbial metabolism are dimethylamine and N-acetyl-Gly (Shi et al., 2018; Li et al., 2024; Suthar et al., 2025). In particular, dimethylamine is a product of microbial fermentation associated with nitrogen metabolism, probably related to protein metabolism (Li et al., 2024; Suthar et al., 2025). In milk, its concentration increases during mastitis (Zhu et al., 2021). These results may reflect increased amino acid metabolism caused by host immune and inflammatory responses and by microbial activity during mammary gland infection.

Moreover, the milk samples of clinical mastitic quarters had greater changes related to microbial activity as evidenced by maltose, arabinose, and formate. The maltose is a disaccharide that reduces in milk of mastitic cows (Zhu et al., 2021). This metabolite can be fermented by *Bacillus* spp., and some *Enterobacteriaceae* such as *Escherichia coli* and *Serratia marcescens* (Munsi et al., 2016; Owusu-Kwarteng et al., 2017; Nair and Devi, 2025). Indeed, the ability of staphylococci and streptococci to ferment maltose can be variable, even if the maltose-negative staphylococci represented 1.46% of bacteria isolated in milk (Karzis et al., 2020; Ilimbayeva et al., 2025). Moreover, the ability of staphylococci to ferment maltose has been proposed to be one of the components that can influence virulence (contagiousness and pathogenicity; Karzis et al., 2020). In this study, the milk maltose level decreased progressively from healthy to clinical mastitis quarters, with 90.6% in IMI, 82.8% in SCM, and 100% in CM of isolated pathogens that can potentially ferment the metabolite, even if phenotypical characterization of strains was not performed in this study. Arabinose is a component of hemicellulose that can be identified directly or through its derivatives in bovine biofluids such as serum and milk (Wendisch et al., 2016; Kim et al., 2021). Reduction in the concentration of this metabolite has been associated with bacterial proliferation, as arabinose isomerase plays an important role in microbial metabolism (Chen and Mu, 2021; Kim et al., 2021). Moreover, arabinose isomerase can also be produced by several bacteria usually present in the udder and consequently in raw milk as *Lactobacillus* spp. and *Bifidobacterium* spp. (Chen et al., 2021; Taye et al., 2021). In this study, the arabinose level decreased only in the CM group. This finding could be related to the *E. coli* infection identified mainly in this group. In fact, *E. coli* can use arabinose as a carbon source for growing (Chen and Mu, 2021). Another compound associated with microbial activity is formate. This metabolite is produced during carbon metabolism, usually by microbial fermentation in the rumen, which also affects its concentration in the blood (Yue et al., 2020; Fiore et al., 2023). In addition, increased milk concentration of formate were observed in milk samples during mastitis (Du et al., 2024; Li et al., 2024). This finding was related to high milk bacteria loads and high SCC level due to the disease (Luangwilai et al., 2021). In our study, the level of formate increased only in the CM groups. This result could be related to the crossing of blood formate through the mammary gland barrier due to inflammation and infection of the mammary gland.

Energy Metabolism

Lactose is secreted by mammary gland epithelial cells into milk from glucose and galactose (Antanaitis et al., 2021). Moreover, milk glycosidases are involved in an inverse reaction (from lactose to glucose and galactose). These enzymes can be leaked into the milk from epithelial or immune cells, as well as from milk contaminating microorganisms. Glycosidase activity has been found to increase in milk from inflamed bovine quarters, with a decrease in lactose and an increase in galactose levels (Sunds et al., 2021). However, generally the concentration of both lactose and its precursors (glucose and galactose) decreases in milk of mastitic cows (Xi et al., 2017; Zhu et al., 2021). Glucose level can be depleted in infectious site by immune cells and microorganisms as energy resource (Xi et al., 2017). However, galactose catabolism can depend on the etiological agents. In fact, *Streptococcus* spp., *Staphylococcus* spp., and *Enterobacteria* including *E. coli* are able to use this monosaccharide (Csiszovszki et al., 2011; Pan et al., 2018; Hiron et al., 2024), and they were mostly found in the bacteriological analysis of the IMI, SCM, and CM groups. In contrast, *Bacillus* spp. cannot catabolize galactose (Wendisch et al., 2016). The milk glucose concentration had a progressive decrease from H to CM quarters of this study, whereas galactose level differed only in the CM group. In addition, milk ribose concentration was decreased only in CM quarters. Ribose is a component of vitamins involved in immune function and energy metabolism as riboflavin (vitamin B_2) and cyanocobalamin (vitamin B_{12}), and it is also involved in glycoconjugate biosynthesis via mono- or diphosphate sugar nucleotides containing adenine (AMP, ADP), guanidine (GDP), or cytidine (CMP; Fox et al, 2015; Mineguchi et al., 2021; Du et al., 2024). Moreover, ribose is involved with the protein family of poly(ADP-ribose) polymerases (PARP) activated during DNA repair, inflammation, mitochondrial oxidation, and

glycolysis (Bai, 2015). Additionally, ribose is a primary fermentable component of RNA by microorganisms (Zeibich et al., 2019). Consequently, this carbohydrate can influence the pentose phosphate pathway, glycolysis, TCA, and the electron transport chain (Soni et al., 2023). These findings could be related to an increased energy metabolism by the host or microorganisms, or both, especially in clinically mastitic quarters with suspected greater inflammation and oxidative stress. Moreover, the hierarchical clustering heatmap revealed distinct metabolomic differences between healthy and infected groups, particularly in CM cases. The metabolite clustering used for pathway analysis evinced that the CM group had greater activity of glycolysis or gluconeogenesis and lower activity of galactose metabolism.

Increased energy metabolism activity may also affect the TCA cycle. Pyruvate represents the starting point for gluconeogenesis and TCA, as the end product of glycolysis. Moreover, it is also related to energy metabolism linked to volatile fatty acids, β-oxidation, and lactate pathway (Sun et al., 2017; Ceciliani et al., 2018). Pyruvate can also be synthetized by Ala as main glucogenic amino acids (Sun et al., 2017). Increase of milk pyruvate, Ala, lactate, and acetate has previously been linked to mastitic condition (Sundekilde et al., 2013; Sunds et al., 2021; Zhu et al., 2021). However, these metabolites are related not only to inflammation but also to microorganism metabolism (Xi et al., 2017). In addition, high milk levels of lactate represent a distinct metabolic fingerprint characterized by presence of bacteria (Zhu et al., 2021). In this study, milk pyruvate, Ala, and acetate levels increased only in the CM group, whereas lactate showed a progressive increase from healthy to mastitic quarters. These findings could represent greater activation of energy metabolic pathways in CM cows, probably related to a greater inflammatory condition and immune response. However, greater activation of the TCA cycle could also lead to a progressive block of itself due to consumption of intermediates (Sun et al., 2014). Instead, lactate concentration could indicate a progressive increase in bacteria presence in IMI, SCM, and CM quarters. In addition, pathway analysis showed a greater activity of pyruvate metabolism, and lower activities of glycine, serine and threonine metabolism, and the TCA cycle in the CM group.

In mitochondria, citrate is one of the first intermediates in the TCA cycle where citrate can be isomerized into *cis*-aconitate (Xu et al., 2020). Milk concentration of citrate decreased at the peak of infection. Consequently, the *cis*-aconitate level is reduced in cows affected by subclinical and clinical mastitis, suggesting a downregulation of the TCA cycle (Xi et al., 2017; Zhu et al., 2021). In our study, *cis*-aconitate had a progressive reduction from H and IMI quarters to CM quarters, in accordance with previous studies. However, the milk metabolome could also be affected by microorganism metabolism (Xi et al., 2017). Fumarate is also an intermediate of the TCA and urea cycles. Consequently, it is related to energy metabolism (Zhu et al., 2021). The fumarate concentration in milk was inversely related to SCC levels, with a decrease in metabolite concentration during mastitis (Sundekilde et al., 2013). This change is associated with energy consumption due to bacterial infection or to impaired TCA function (Zhu et al., 2021). The milk fumarate level decreased in SCM and CM quarters of this study, which could be related to the SCC cutoff applied for group division. Therefore, impaired TCA activity could also be suspected during subclinical mastitis, even if the milk of CM quarters showed more alterations in metabolites linked to energy metabolism (pyruvate, Ala, acetate, and *cis*-aconitate). However, this change could be useful, considering that increased levels of fumarate and its derivates are associated with increased persistence of antibiotic-susceptible bacteria such as *E. coli* and *S. aureus* in the presence of antibiotics (Lin et al., 2024). The progressive increment of energy requirement due to inflammatory response and infection in the SCM and CM groups can also be suggested by the 2-ketobutyrate level. This metabolite derives from Thr catabolism to produce propionyl-CoA and energy production by TCA (Coleman et al., 2020). In fact, 2-ketobutyrate decreased progressively in SCM and CM samples.

Creatine-phosphate, or phosphocreatine, represents an energy resource to produce ATP, especially in muscle tissue (Sun et al., 2017; Ceciliani et al., 2018). This metabolite has previously been found in milk, via NMR, with a range of 0.585 to 2.567 mmol/L (Klein et al., 2010). Furthermore, its concentration tends to decrease during mastitis (Zhu et al., 2021) as in this study, even if identified levels were well below the previously mentioned range (0.076–0.167 mmol/L) in all groups. Considering the damage to the milk barrier with a flow of metabolites from blood to milk (Zhu et al., 2021), the reduced milk creatine-phosphate concentrations during subclinical and clinical mastitis could represent an indication of poor systemic energy status, as hypothesized in a blood metabolomic study during subclinical mastitis (Lisuzzo et al., 2024). Moreover, phosphocreatine may also be related to the pyruvate level. In fact, an elevated concentration of the latter inhibited creatinine-pyruvate kinase in humans, with a consequent reduction of phosphocreatine (Ceciliani et al., 2018).

Lipid Metabolism

A reduced activity of glycerophospholipid metabolism in CM quarters was discovered during the pathway analysis. Impaired lipid metabolism in milk is mainly related

to phospholipid, glycerophospholipid, and carnitine metabolisms (Li et al., 2024). Choline is a constituent of phosphocholine, which is metabolized subsequently in citicoline, phosphatidylcholine, and glycerophosphocholine (Xu et al., 2020; Haxhiaj et al., 2022). All of these are structural components of cell membrane (Xi et al., 2017; Kong et al., 2021; Li et al., 2024). Moreover, the ratio between glycerophosphocholine and phosphocholine has been associated with cell membrane synthesis during cell proliferation, characterized by high concentrations of phosphocholine (Xu et al., 2020). In fact, hypoxic condition of mammary gland tissue has been associated with lower levels of choline and citicoline, and greater level of glycerol-3-phosphate, a constituent of glycerophosphocholine (Kong et al., 2021). Reduced milk levels of glycerophosphocholine and phosphocholine have previously been associated with mastitis conditions, probably due to destruction of the cell membrane of the mammary tissue (Xi et al., 2017; Li et al., 2024). In our study, the H group was characterized by greater levels of O-phosphocholine and citicoline, and a lower level of sn-glycerol-3-phosphocholine (considering the identified phosphocholine [**PC**] and glycerophosphocholine [**GPC**] of the untargeted analysis of this trial, the GPC:PC ratio = 5.91); the IMI group was characterized by greater levels of these metabolites (GPC:PC = 9.32); the SCM group showed an opposite trend compared with H, with a greater level of sn-glycerol-3-phosphocholine, and lower levels of O-phosphocholine and citicoline (GPC:PC = 11.83); and the CM group had lower levels of these metabolites (GPC:PC = 7.26). These results could suggest an increase in cell proliferation probably related to the immune response, particularly in the IMI and SCM groups, with a shift from phosphocholine to glycerophosphocholine concentration and progressive consumption of substrates in the CM group. Additionally, choline can be oxidized in betaine for methyl donor activity and carnitine biosynthesis (Peterson et al., 2012; Zandkarimi et al., 2018). However, betaine was greater in the IMI group only compared with the H group, suggesting that its role in mammary infection and inflammation might only be initial. Carnitine is involved in long-chain fatty acid transport from cytosol to the mitochondrial matrix, consequently influencing fatty acid oxidation and the TCA cycle (Xi et al., 2017; Zhu et al., 2021). This metabolite has been identified in the mammary gland tissue only of lactating cows, probably related to its function (Sun et al., 2017). In milk, the decrease in carnitine concentration has previously been associated with both subclinical and clinical mastitis, and consequently with inflammation, infection, and immune function (Sen et al., 2021; Sunds et al., 2021; Du et al., 2024). For these reasons, it has been hypothesized that lipid and energy metabolism is downregulated during mastitis (Li et al., 2024). However, the role of bacterial species must be considered for milk samples of mastitic cows. In fact, carnitine can be acquired by bacteria for their metabolism, but their ability to synthesize it has not been demonstrated (Meadows and Wargo, 2015). In this study, carnitine levels decreased in subclinical mastitis samples and further in CM. This result may be associated with reduced energy metabolism, which negatively affects the immune and inflammatory responses, or with metabolite consumption by microorganisms present during infection.

Ketone Body Metabolism

Reduction of the TCA cycle is related to increased production of ketone bodies. In fact, ketone bodies can be used as an alternative energy resource for local immune and inflammatory responses during mastitis (Lisuzzo et al., 2022, 2024). In the present study, 2 ketone bodies were identified in milk: acetoacetate, and 3-hydroxybutyrate or BHB. The increase of these metabolites has previously been associated with mastitis (Zhu et al., 2021; Li et al., 2024). However, they had an opposite trend in the present study, with a reduced concentration of acetoacetate and an increased concentration of BHB in CM. This condition could be linked to the progressive synthesis of BHB, which is preceded by acetoacetate, for altered TCA functioning, or to the influence of microorganisms that use these energy substrates. Moreover, the BHB level is also influenced by butyrate derived by ruminal fermentation (Johnzon et al., 2018). Ruminal production of volatile fatty acids (acetate, propionate, and butyrate) is reduced during clinical mastitis, with a consequent potential negative effect on milk fat synthesis (Wang et al., 2021). The butyrate level decreased only in the SCM group milk samples of this study, probably related to impaired ruminal fermentation. However, the butyrate level in the CM group was similar to that of the H group. The greater level of this volatile fatty acid during clinical mastitis could have also influenced milk BHB concentration. In addition, this finding could also be associated with impaired fatty acids synthesis during inflammatory conditions, with a possible reduced consumption of butyrate.

The branched-chain amino acids (**BCAA**), such as Leu and Val, can be used to produce energy through the TCA cycle or to produce keto acids (ketogenic amino acids; Dervishi et al., 2017). Moreover, BCAA are an important substrate for protein synthesis by mammary tissue for milk protein, and by immune cells, consequently affecting the immune response (Coleman et al., 2020; Huang et al., 2021). However, Leu and Val can also be used by bacteria for the same reason. Consequently, increased milk BCAA levels are indicators of immune function

and mammary inflammation and infection (Grispoldi et al., 2019; Zhu et al., 2021; Du et al., 2024). In addition, Val and Leu are involved in control of the redox state, increasing defenses against oxidative stress (Dervishi et al., 2017). In our study, both amino acids were increased only in the CM group, probably related to greater inflammation and immune responses, and their increased level could also be related to changes in energy metabolism, considering that both Leu and Val are ketogenic amino acids.

Oxidative Stress

Aminoadipic acid is a product of Lys catabolism to produce acetyl-CoA, and consequently energy trough the TCA cycle (Kohlmeier, 2015; Zhu et al., 2020). Increased concentration of this metabolite reflects an increased energy demand by Lys oxidation (Ghaffari et al., 2025). Moreover, the increment is also considered an indicator of oxidative stress in humans (Estaras et al., 2020). 2-Aminoadipate was identified in this study with progressively increased concentrations in the IMI, SCM, and CM groups. This finding could further represent an increment of energy requirements for inflammatory and immune responses during mammary gland infection in addition to a progressive increment of oxidative stress. Instead, taurine and ascorbate represent 2 antioxidants. Taurine is an intracellular amino acid with anti-inflammatory, antioxidative, and immunomodulate activity. It is synthesized especially in hepatic and brain tissues, but mammary duct epithelial cells, stromal adipocytes in the mammary gland, and adipose tissue also take part in it (Coleman et al., 2020; Li et al., 2021; Torunoglu and Yarim, 2024). In vitro, taurine administration to mammary epithelial cells attenuated epithelial damage and inflammatory response, while an in vivo study on mice suggested that taurine could protect the blood-milk barrier during mastitis (Li et al., 2021; Liu et al., 2024). In addition, ascorbate, or vitamin C, is the most abundant antioxidant for mammals, acting as a scavenger of reactive oxygen species by rapid electron transfer, thus inhibiting lipid peroxidation. Both plasma and milk ascorbate level were decreased during mastitic conditions, with a suggested link between the decrease in vitamin C concentration and the severity of clinical signs (Ranjan et al., 2005; Yang and Li, 2015). In our study, milk taurine level increased only in the CM group, whereas ascorbate concentration decreased in the same one. These findings could be related to a greater oxidative stress in clinical mastitis with reduction of ascorbate level. At the same time, the increase in taurine concentration could suggest leakage by mammary cells or a greater synthesis by the liver in response to inflammatory conditions to manage it. Phenylacetate concentration decreased in all mastitis milk samples (IMI, SCM, and CM) compared with healthy quarters. This metabolite derives from deamination of aromatic amino acids such as Tyr and Phe (Suthar et al., 2025). Aromatic amino acids are important components for NO production in macrophages, thus influencing immune response (Dervishi et al., 2017; Coleman et al., 2020; Du et al., 2024). The finding of this study could be related to consumption of aromatic amino acids by immune cells with consequent decrease in the phenylacetate level, or to a reduced deamination of them. However, aromatic amino acids were not identified in milk samples, and consequently a complete evaluation cannot be performed.

Strengths, Limitations, and Future Prospects

Carbohydrates, fats, and amino acids are the basis of every cellular process, including inflammatory and immune processes. An immune response by the body, including the mammary gland, involves cytokines, lactoferrin, transferrin, lysozyme, acute phase proteins, antimicrobial peptides, and antibodies, to which receptors are added. Proteins and enzymes are made up of amino acids. Therefore, these compounds can influence a proper inflammatory and immune response (Kohlmeier, 2015; Haxhiaj et al., 2022). Furthermore, the activation of inflammatory and immune responses increases energy demand, leading to the use of carbohydrates and fatty acids, with an estimated consumption of over 1 kg of glucose in 12 h of inflammatory activation (Kvidera et al., 2017). Amino acids can also be included in this energetic context, as they are also energy precursors (Kohlmeier, 2015). For this reason, an evaluation of metabolites during mastitis can help to better contextualize the pathological condition. However, the milk metabolome evaluation must also consider the metabolites produced by microorganisms. Therefore, the metabolome can be influenced by the specific etiological pathogen involved in mastitis (Du et al., 2024; Lisuzzo et al., 2024). The present study did not aim to discern the evaluated udder health status (H, IMI, SCM, and CM) in relation to a specific pathogen. This choice represents a field condition, but at the same time it can also be a limitation of the study. Further studies evaluating the same health states for specific etiological pathogen are therefore necessary for a more accurate assessment of the milk metabolome. Furthermore, to the best of the authors' knowledge, this study was the first to evaluate a condition of udder infection in the absence of elevated SCC. The mastitis monitoring program applied in the study allowed for the enrollment of quarters that were not previously diagnosed with mastitis, thus avoiding the inclusion of chronic conditions in the study. However, the study design was cross-sectional. For this reason, the

mastitis evolution after the trial was missing, representing another study limitation. Finally, the knowledge of the milk metabolome in these udder health statuses (H, IMI, SCM, and CM) may represent a starting point for future studies evaluating therapy efficacy.

CONCLUSIONS

The milk metabolomic profiles identified in this study were affected in animals with different udder health statuses. The changes identified suggested progressive increase in inflammatory status, damage to the blood-milk barrier, oxidative stress, and microorganism metabolism. In addition, an increase in cell proliferation related to the immune response was suspected during intramammary infection and subclinical mastitis, with progressive substrate consumption in clinical mastitis. Furthermore, both subclinical and clinical mastitis showed impaired energy metabolism due to suspected reduced functioning of the TCA cycle and lipid metabolism, as well as altered systemic energy status with worse conditions during clinical mastitis. The results of this study showed that metabolomic alterations begin to occur in conditions of mammary gland infection with somatic cells within the healthy range. Further studies comparing the metabolomic profile of blood and milk could be useful to better characterize animal health status.

NOTES

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Nonstandard abbreviations used: AIC = Akaike information criterion; BCAA = branched-chain amino acids; BIC = Bayesian information criterion; BMC = bacteriological milk cultures; CM = clinical mastitis group; CMT = California Mastitis Test; GPC = glycerophosphocholine; H = healthy group; KEGG = Kyoto Encyclopedia of Genes and Genomes; NAGase = N-acetylglucosaminidase; NMR = nuclear magnetic resonance; PC = phosphocholine; SCM = subclinical mastitis group; sPLS-DA = sparse partial least squares discriminant analysis; TCA = citrate cycle; TSP = 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid-sodium salt.

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ORCIDS

A. Lisuzzo, https://orcid.org/0000-0001-7064-0749
M. C. Alterisio, https://orcid.org/0000-0002-0101-0783
L. Laghi, https://orcid.org/0000-0003-1593-7376
A. Pesce, https://orcid.org/0000-0003-2256-0538
P. Ciaramella, https://orcid.org/0000-0002-5518-5378
M. Berlanda, https://orcid.org/0000-0001-5961-0987
M. Gianesella, https://orcid.org/0000-0001-8117-5969
J. Guccione, https://orcid.org/0000-0002-3390-137X
E. Fiore https://orcid.org/0000-0002-0377-9553