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Serum metabolome differences associated with subclinical intramammary infection caused by *Streptococcus agalactiae* and *Prototheca* spp. in multiparous dairy cows

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ABSTRACT

Mastitis is one of the most significant diseases in dairy cows and causes several economic losses. Somatic cell count (SCC) is often used as an indirect diagnostic tool for mastitis, especially for subclinical mastitis (SCM) where no symptoms or signs can be detected. *Streptococcus agalactiae* is one of the main causes of contagious mastitis, and *Prototheca* spp. is an alga-inducing environmental mastitis that is not always correlated with increased milk SCC. The aim of this study was to evaluate the changes in the metabolomic profile of blood in relation to subclinical intramammary infection (IMI) in dairy cows. In addition, differences resulting from the etiologic agent causing mastitis were also considered. Forty Holstein-Friesian dairy cows in mid and late lactation were enrolled in this cross-sectional design study. Based on the bacteriological examination of milk, the animals were divided into 3 groups: group CTR (control group; n = 16), group A (affected by SCM with IMI caused by *Strep. agalactiae*; n = 17), and group P (affected by SCM with IMI caused by *Prototheca* spp.; n = 7). Blood samples from the jugular vein were collected in tubes containing clot activator; the serum aliquot was stored until metabolomic analysis by ^{1}H-nuclear magnetic resonance spectroscopy. Statistical analysis was conducted by fitting a linear model with the group as the fixed effect and SCC as the covariate. Forty-two metabolites were identified, and among them 10 were significantly different among groups. Groups A and P showed greater levels of His and lactose and lower levels of acetate, Asn, and dimethylamine compared with group CTR. Group A showed high levels of Val, and group P showed high levels of Cit and methylguanidine, as well as lower levels of 3-hydroxybutyrate, acetone, allantoin, carnitine, citrate, and ethanol. These metabolites were related to ruminal fermentations, energy metabolism, urea synthesis and metabolism, immune and inflammatory response, and mammary gland permeability. These results suggest systemic involvement with subclinical IMI and that the metabolic profile of animals with SCM undergoes changes related to the etiological agent of mastitis.

Key words: mastitis, metabolomics, *Streptococcus agalactiae*, *Prototheca* spp., dairy cows

INTRODUCTION

Mastitis is one of the most important diseases in dairy cows because it is associated with decreased milk production, higher culling rates and drug costs, and decreased quality of dairy products. This inflammatory disease of the mammary glands can be caused by microorganisms, usually bacteria, that induce tissue damage or, less frequently, by chemical, physical, or traumatic accidents (Carvalho-Sombra et al., 2021).

Based on epidemiology, mastitis is classified as either contagious or environmental. Contagious mastitis is caused by bacteria, including *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Mycoplasma* spp., which spread between animals, usually at the time of milking, through contaminated hands, towels, or the milking machine acting as bacterial reservoirs. Environmental mastitis is caused by bacteria that originate from the environment and spread mainly through bedding, soil, feces, and standing water (Sharun et al., 2021). Once the mammary glands have been infected, PMN are recruited in situ by resident cells (lymphocytes, macrophages, and epithelial cells) resulting in an increased SCC. Subsequently, PMN tend to be reduced in favor of macrophages that eliminate bacteria and debris (Halasa and Kirkeby, 2020). It is hypothesized that the severity and duration of mastitis are highly correlated with the promptness of the leukocyte migratory response

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and the bactericidal activity of immune cells at the site of infection. If cells move rapidly from the blood to the mammary gland and eliminate bacteria, cell recruitment ceases. However, SCC during the chronic phase may fluctuate but generally remains elevated (Halasa and Kirkeby, 2020). For these reasons, SCC is often used as an indirect diagnostic tool for mastitis; a threshold count of 200,000 cells/mL is indicative of mastitis (Pegolo et al., 2022). *Streptococcus agalactiae* is a major cause of contagious subclinical mastitis (**SCM**) worldwide. Indirectly, the bacterium can activate the cow's immune system, causing an influx of components from the blood into the milk. Conversely, alga-inducing *Prototheca* spp. cause chronic granulomatous interstitial environmental mastitis, which is not always associated with increased milk SCC (Åkerstedt et al., 2012; Wawron et al., 2013).

Depending on the signs and severity, mastitis can be classified as clinical mastitis (**CM**) or SCM. Clinical mastitis can be classified as mild, moderate, or severe. A change in milk color and consistency is present in mild CM. Mammary gland changes, including redness, swelling, warmth, and pain, occur in moderate CM. The presence of systemic signs such as fever, loss of appetite, and inability or reluctance to move are suggestive of severe CM. However, SCM has no signs and can spread through the herd without detection. The only way to detect it is an increase in milk SCC above the cut-off previously indicated (Haxhiaj et al., 2022b).

Metabolomics is the large-scale study of metabolites. One of its primary applications is the identification of metabolites that are associated with health status. This tool is used to understand the underlying metabolic changes associated with the transition period in dairy cows and how it relates to disease manifestation (Lisuzzo et al., 2022c). In addition, metabolomics can identify potential metabolite biomarkers to detect animals susceptible to various diseases, including mastitis (Haxhiaj et al., 2022b). Blood represents a primary carrier that contains metabolites connected to all organs of the body. Therefore, metabolites present in serum or plasma may be subject to cellular uptake and metabolism and can be used by different tissues, such as the mammary gland (Lisuzzo et al., 2022c; Fiore et al., 2023). Nuclear magnetic resonance (**NMR**) spectroscopy and MS are the most commonly applied platforms for metabolomics analysis. The latter shows higher sensitivity and the possibility to identify unknown compounds. In addition, this platform is usually integrated with separation techniques such as GC, liquid chromatography, or capillary electrophoresis. However, necessary sample preparation, such as extraction and derivatization, can lead to loss of metabolites or discriminate against specific classes of metabolites (e.g., only volatile and thermostable metabolites can be identified in GC-MS). The NMR technology is based on magnetic properties (i.e., a spin that generates a small, local magnetic field) characteristic of certain nuclei of atoms (e.g., ^{1}H, ^{13}C, ^{31}P, and others) generating chemical information; ^{1}H-NMR spectroscopy is one of the best-known platforms for metabolomics because hydrogen is naturally abundant and present in almost all organic molecules. Despite the lower sensitivity of NMR compared with MS, this platform has multiple positive attributes: simple sample preparation, simultaneous detection of a wide spectrum of metabolite classes, non-destructivity, and high reproducibility. The analysis may be targeted or untargeted in both NMR and MS. Targeted metabolomics quantifies biochemically known and chemically characterized metabolites derived by a targeted sample preparation, and non-targeted metabolomics is a global unbiased analysis of as many measurable metabolites as possible within a sample. The resulting outcomes are used to create metabolome databases. The most comprehensive and exhaustive is the human metabolome database (**HMDB**). Its bovine-specific counterpart is under development and is not yet included in the most widely used metabolomics software (MetaboAnalyst; Zhang and Ametaj, 2020; Fiore et al., 2020).

In this study, we hypothesized that subclinical IMI (**sIMI**) caused by *Strep. agalactiae* and *Prototheca* spp. is associated with changes in blood metabolomic profile in dairy cows. Furthermore, to the best of our knowledge, potential pathogen-specific effects have not been investigated. Therefore, the aim of this study was to investigate the changes in the metabolomic profile of blood upon sIMI from *Strep. agalactiae* and *Prototheca* spp. in Holstein dairy cows.

MATERIALS AND METHODS

Animals and Study Design

This study was part of the University of Padua Department of Animal Medicine, Production, and Health's LATSAN project, which aimed to develop innovative tools for evaluating and studying mammary gland health and improving dairy cows' nutritional milk quality and coagulation properties. Originally, 188 Holstein-Friesian dairy cows located on one commercial farm in Italy's Veneto region were selected based on clinical examination by veterinarians of the University Veterinary Teaching Hospital (OVUD), University of Padua, and on the following criteria: (1) multiparous, (2) more than 120 DIM, (3) absence of clinical signs of disease, and (4) no antibiotic treatment or anti-inflammatory medications before enrollment. Moreover, negative control animals had no previous history of mastitis.

The farm was selected on the basis of herd prevalence of *Strep. agalactiae,* and *Prototheca* spp. based on an initial survey carried out by the Istituto Zooprofilattico Sperimentale delle Venezie (**IZSVe**) and, specifically for this study, the choice was made according to the simultaneous and well-balanced presence of the 2 pathogens (i.e., *Strep. agalactiae* and *Prototheca* spp.) thus allowing us to test the hypothesis and make inferences within the herd. All cows were housed in freestalls and were fed a TMR based mainly on corn silage, sorghum silage, and concentrates (Supplemental Table S1; https://data.mendeley.com/datasets/vf65kjf75b/1). Drinking water was ad libitum, and cows were milked twice daily. All animals periodically received a clinical examination by veterinarians, and animals with clinical signs of disease or under medical treatment were excluded from this study.

A first bacteriological screening on milk (n = 64) was conducted, and the results were confirmed 2 wk later. Only animals that confirmed the bacteriological results from the initial screening to the follow-up screening and without sample contamination were selected for blood metabolomics analysis using a cross-sectional design. Three groups were then formed: the control group (**group CTR**; n = 16), cows affected by SCM with IMI caused by *Strep. agalactiae* (**group A**; n = 17), and cows affected by SCM with IMI caused by *Prototheca* spp. (**group P**; n = 7). The average DIM of the selected animals was 237 ± 21 d (217 d for group CTR, 263 d for group A, and 231 d for group P).

Pooled milk samples were aseptically collected following National Mastitis Council guidelines (NMC; 2017). Briefly, teat ends were cleaned externally with commercial pre-milking disinfectants by the veterinarian, dried with individual towels, and then cleaned again with alcohol. After discarding the first streams of foremilk, approximately 10 mL of milk from each quarter was collected in sterile tubes and pooled. Blood sampling was carried out the same day. Samples were collected from the jugular vein with an evacuated tube system for each enrolled cow. The samples were stored in Venosafe tubes (9 mL; Terumo Venosafe, Leuvel, Belgium) containing clot activator. Both milk and blood samples were refrigerated at 4°C and transported in a portable freezer (CoolFreeze CFX65 W professional, Dometic, Stockholm, Sweden; minimum temperature −22°C) at the same constant temperature to the laboratory of the Department of Animal Medicine, Production and Health (MAPS) of the University of Padua within 1 h of sampling. Blood samples were centrifuged at 1,750 × *g* for 10 min at room temperature (20°C; Heraeus Labofouge 400, Thermo Scientific, Milan, Italy). One aliquot of serum was obtained from each cow and placed in a 1.5-mL Eppendorf tube. The serum aliquot was stored at −80°C until non-targeted metabolomic analysis. One aliquot of milk sample for each animal was delivered to IZSVe for microbiological analysis as mentioned by Pegolo et al. (2022). Briefly, 10 μL of milk was inoculated on each of the following selective media: Tallium Kristalviolette Tossin agar (**TKT**; IZSVe internal production) and *Prothoteca* spp. isolation medium (**PIM**; IZSVe internal production). The method described by Hauge and Ellingsen (1953) was used to prepare TKT plates, and PIM plates were prepared according to the National Mastitis Council guideline (NMC, 2017). Putative colonies of *Strep. agalactiae* were observed on the TKT agar plates at 24 h and confirmed using the Christie–Atkins–Munch-Peterson (CAMP) test (NMC, 2017). At 24 h, 48 h, and 72 h the PIM plates were observed and putative colonies were confirmed using a wet mount method (NMC, 2017). The second aliquot of milk sample was used for the analysis of milk composition (protein, casein, fat, and lactose as percent, and urea as mg/100 g) using an FT6000 Milkoscan infrared analyzer (Foss A/S, Padua, Italy). The SCC (cells/mL) and differential SCC were determined using a Fossomatic 7 DC analyzer (Foss A/S).

Metabolomic Analysis

The non-targeted metabolomics investigation was carried out using an NMR analysis solution with 10 m*M* 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt (TSP; chemical-shift reference) in D_2O set at pH 7.00 ± 0.02 by means of 1 *M* phosphate buffer containing 2 m*M* NaN_3. The NaN_3 was used to avoid microbial proliferation, as suggested by Zhu et al. (2020). Serum samples were prepared for ^{1}H-NMR spectroscopy by thawing and centrifuging 1 mL of each sample for 15 min at 18,630 × *g* and 4°C. The supernatant (700 μL) was added to 100 μL of NMR analysis solution. Finally, each sample was centrifuged as previously mentioned. 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. According to Zhu et al. (2020), the signals from broad resonances originating from large molecules were suppressed by a Carr-Purcell-Meiboom-Gill (CPMG) filter comprised of 400 echoes with a τ of 400 μs and a 180° pulse of 24 μs for a total filter of 330 ms. The water residual signal was suppressed by means of a presaturation technique. This setting employed the cpmgpr1d sequence, which is 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 5 s. The spectral phase was manually adjusted in Topspin, with subsequent

adjustments performed in R computational language by means of a script developed in-house (R Core Development Team, 2018). After the removal of the residual water signal, the ^{1}H-NMR spectra were baseline-corrected by means of peak detection, according to the "rolling ball" principle (Kneen and Annegarn, 1996), implemented in the baseline R package (Liland et al., 2010). Each signal was assigned in an untargeted manner by comparing its chemical shift (the position along the spectrum) and multiplicity (the shape of the signal) through the software Chenomx (ver 8.3, Chenomx Inc., Edmonton, AB, Canada) with Chenomx (ver 10, 336 compounds) and with HMDB (ver 2, 643 compounds) databases. This was done by superimposing the registered spectra with spectra obtained on pure compounds constituting the databases. The molecules of the first serum sample analyzed were quantified by means of an external standard by taking advantage of the principle of reciprocity (Hoult, 2011). Differences in water content among samples were then taken into consideration by probabilistic quotient normalization (Dieterle et al., 2006). Molecule quantification was performed by means of rectangular integration, considering one of the corresponding signals free from interferences.

Statistical Analysis

The minimum sample size was calculated a priori using G*Power software v. 3.1.9.7 (http://www.gpower.hhu.de/). This analysis used the value of SCC as a discriminant between control and subclinical mastitic animals presented by Haxhiaj et al. (2022a) using an α of 0.05 and a β of 0.20. The minimum sample size found was 6 animals per group.

Statistical analysis was performed with R software v. 4.2.1 (https://www.R-project.org/). The normal distribution of data was confirmed by using the Shapiro–Wilk test. Considering the hypothesis of an influence of sIMI on changes in blood metabolomic profile in dairy cows, and the potential pathogen-specific effects, the following linear model was selected to assess the difference between groups:

$$y_i = \mu + Group + SCC + e_i,$$

where y_i is the investigated phenotype previously defined (i.e., milk composition and serum metabolites); μ is the overall mean; and *Group* is the fixed effect (3 groups: CTR; group A, or group P). Considering fluctuations in SCC levels related to mastitis presentation and the specific pathogen involved, as previously explained, this parameter (SCC) was used as a covariate describing its effect on phenotype. The e_i is the random residual. A post hoc pairwise comparison of the least squares means were performed using Bonferroni correction. The data were expressed as LSMEANS and SEM. The significance threshold was set at $P \leq 0.05$. Parameters that presented a P-value between 0.05 and 0.1 were considered trends of significance.

To study the metabolic pathways influenced by serum metabolome associated with a group and to evaluate the biological function of metabolites, the MetaboAnalyst 5.0 software (https://www.metaboanalyst.ca) was used. A hierarchical clustering heatmap was generated with autoscaling data (mean-centered and divided by the standard deviation of each variable) to identify metabolome differences between groups. Based on the differences among metabolites' profiles, clusters and subclusters were identified, and the lists of their metabolites were used for pathway analysis. The pathway analysis was used to assess the metabolic pathways influenced by mammary health status using the *Bos taurus* selected library. 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. A principal component analysis (**PCA**), a partial least squares-discriminant analysis (**PLS-DA**), and an orthogonal partial least squares-discriminant analysis (**OPLS-DA**) were generated with autoscaling data (mean-centered and divided by the standard deviation of each variable) using animals affected by sIMI versus group CTR, and group A versus group P to highlight the trends of serum metabolome. The PCA analysis is an unsupervised method generally used as an exploratory clustering technique to assess differences between observations. This analysis was followed by PLS-DA and OPLS-DA, 2 supervised methods that progressively maximize the separation between groups by eliminating potential errors. The variable importance in projection scores was applied to PLS-DA and OPLS-DA analysis to identify the metabolites contributing the most to variance between groups.

Using the MetaboAnalyst 5.0 software (https://www.metaboanalyst.ca), the websites of PubChem (https://pubchem.ncbi.nlm.nih.gov/), HMDB (https://hmdb.ca/metabolites/), and Kyoto Encyclopedia of Genes and Genomes (https://www.genome.jp/kegg/) were consulted to assess the function of significant and not significant metabolites (Shi et al., 2021).

RESULTS

Milk composition is described in Table 1. Regarding the SCC, a difference was evidenced among all groups (P <0.001) with the greatest value in group A (4,066.3 $\times$ 10^3 mL^{-1}) and the greater value in group P (1,418.4 $\times$ 10^3 mL^{-1}) compared with group CTR (85.8 $\times$ 10^3

Table 1. Results from ANOVA (*F*-values and *P*-values) for milk composition[1]

Parameter	Group CTR (n = 16)	Group A (n = 17)	Group P (n = 7)	*F*-value	*P*-value	SCC covariate	
						F-value	*P*-value
SCC, 10^3/mL	85.8[c]	4,066.3[a]	1,418.4[b]	13.36	<0.001	—	—
DSCC,[2] %	58.9	68.5	61.4	1.13	0.336	1.71	0.202
Fat, %	2.33	2.21	1.72	1.75	0.192	0.88	0.355
Protein, %	3.43	3.57	3.45	0.36	0.701	0.26	0.614
Lactose, %	4.55	4.80	4.42	2.84	0.076	30.01	<0.001
Casein, %	2.70	2.83	2.66	0.66	0.525	1.78	0.194
Urea, mg/100 g	18.9[b]	26.2[a]	23.5[a]	4.06	0.028	5.35	0.028

[a–c]Mean values in the same row which differ significantly. The significance threshold was set at $P \leq 0.05$. A *P*-value between 0.05 and 0.1 was considered as a trend.

[1]Characterization of dairy cows categorized as control (group CTR; n = 16), infected by *Streptococcus agalactiae* (group A; n = 17), and infected by *Prototheca* spp. (group P; n = 7).

[2]Differential somatic cell count.

mL^{-1}). In contrast, milk urea showed an increase in the sIMI groups compared with group CTR ($P = 0.028$). A significant influence of the covariate was assessed for milk lactose and urea with a negative slope of the SCC ($P < 0.001$ and 0.028, respectively).

The hierarchical clustering heat map identified 2 main clusters of metabolites. The first cluster showed a higher concentration of metabolites in the CTR group than in the sIMI animals. The second cluster can be subdivided into 3 subclusters: the first subcluster showed a greater concentration of metabolites in group P compared with group A; the second subcluster showed a greater concentration of metabolites in group A compared with group P; and the third subcluster showed a higher concentration of metabolites in animals affected by sIMI compared with the CTR group (Figure 1).

The metabolite lists of the first main cluster and the 3 subclusters were used to identify metabolic pathways influenced by the health status of the mammary gland. The main cluster identified influenced the aminoacyl-tRNA biosynthesis (Holm *P*-value = 0.002; Phe, Ala, Ile, Thr, and Tyr) and phenylalanine, tyrosine, and tryptophan biosynthesis (Holm *P*-value = 0.034; Phy and Tyr) pathways. The first subcluster identified the aminoacyl-tRNA biosynthesis (Holm *P*-value = 0.010; Gln, Met, and Leu) pathway, whereas the third subcluster identified the glycine, serine and threonine metabolism (Holm *P*-value = 0.003; choline, betaine, Gly, and glyoxylate) and aminoacyl-tRNA biosynthesis (Holm *P*-value = 0.014; His, Gly, Val, and Pro) pathways. The second subcluster was not able to significantly influence a metabolic pathway (Figure 2).

The PCA analysis was first conducted as an unsupervised method to identify whether different groups segregate together. However, the groups did not clearly cluster according to this analysis. Therefore, PLS-DA and OPLS-DA were performed to progressively maximize the separation between groups (Figure 3A, 3C, 3E, and 3G). Based on a progressive increase of the variable importance in projection score above 1.5 in OPLS-DA, the most important metabolites for group separation were betaine, acetate, Cit, choline, and lactose to discriminate between the sIMI and CTR groups (Figure 3D), and methyl-guanidine, lactate, acetone, Gln, ethanol, and Cit to discriminate between group A and group P (Figure 3H).

A total of 42 metabolites were identified by metabolomic analysis with ^{1}H-NMR spectroscopy. Ten of the quantified metabolites were significantly different among groups: 3-hydroxybutyrate ($P = 0.004$), acetate ($P = 0.013$), acetone ($P = 0.003$), allantoin ($P = 0.016$), Asn ($P = 0.034$), carnitine ($P = 0.039$), Cit ($P = 0.001$), ethanol ($P = 0.013$), lactose ($P = 0.003$), and methylguanidine ($P = 0.009$). Four of the quantified metabolites tended to be significant: citrate ($P = 0.057$), dimethylamine ($P = 0.070$), His ($P = 0.053$), and Val ($P = 0.081$). The metabolite concentrations that differed or tended to differ among groups are listed in Table 2, whereas the concentrations of the remaining metabolites that did not differ are shown in Supplemental Table S2 (https://data.mendeley.com/datasets/vf65kjf75b/1). All statistical differences between metabolite concentrations had a power of at least 80% at post hoc analysis. The fold changes of the representative metabolites are listed in Supplemental Table S3 (https://data.mendeley.com/datasets/vf65kjf75b/1). The SCC was associated only with allantoin and Asn and showed a significant influence, with a positive relationship for Asn ($P = 0.018$) and a negative one for allantoin ($P = 0.009$).

DISCUSSION

The main aim of this study was to evaluate whether sIMI could influence the metabolomic profile of the

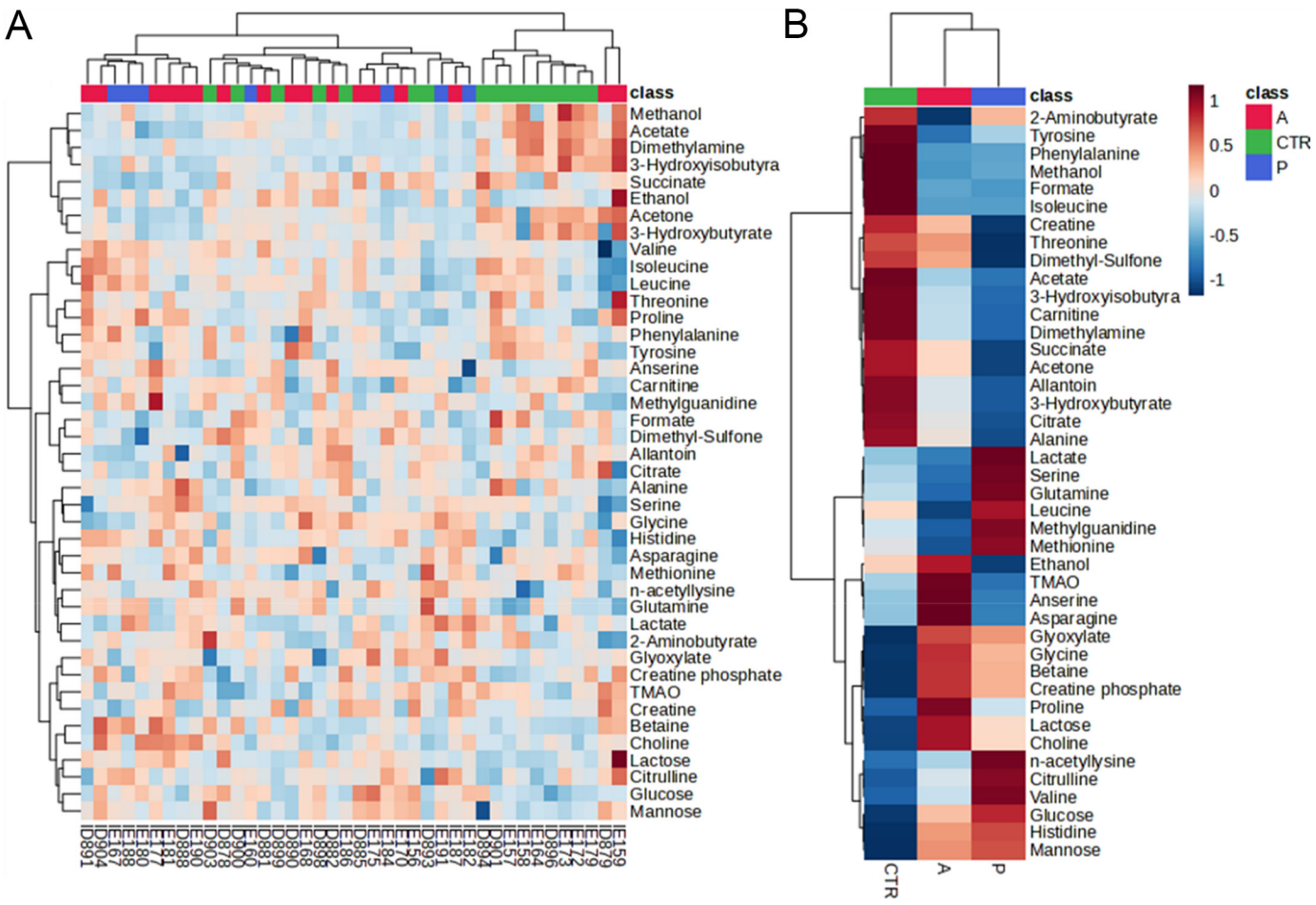

Figure 1. Hierarchical clustering heatmap of all animals (A) and as average of group (B) of all identified metabolites. On the top right is the colorimetric scale: if the color tends to dark red, then the metabolite's concentration was increased; if the color tends to dark blue, then the metabolite's concentration was decreased. The red, green, and blue boxes on the upper right correspond to group CTR (control; n = 16; green), group A (infected by *Streptococcus agalactiae*; n = 17; red), and group P (infected by *Prototheca* spp.; n = 7; blue) and are represented in the first line of each heatmap. On the left side of the heatmaps is the clustering division. Results obtained by the cluster analysis performed with MetaboAnalyst 5.0 (https://www.metaboanalyst.ca).

serum. We also considered whether the etiologic agents causing mastitis selectively influenced the metabolomic profile. Because a cross-sectional design was applied in this study, the animals were sampled only one time. Consequently, data on how the metabolome changed over time from pre-infection to infection is not provided. In addition, only SCM caused by *Strep. agalactiae* and *Prototheca* spp. was considered; differences resulting from other pathogens or disease severity cannot be provided. Therefore, further studies evaluating metabolome changes during the development of CM are needed.

Mastitis alters milk composition and the level of SCC. However, the degree of changes depends on the severity of the inflammatory response, the amount of infected tissue, and the bacterial pathogenicity (Malek dos Reis et al., 2013). In this study, milk composition was altered only in urea concentration, but this result may be because of the low severity of the ongoing mastitis. Blood and milk urea are closely related to one another, and an increase of blood urea in mastitic cows was found using metabolomics analysis (Spek et al., 2016; Dervishi et al., 2017; Gross et al., 2020). Moreover, a decrease in milk urea has generally been found during mastitis (Nyman et al., 2014; Timonen et al., 2017; Wang et al., 2020). The increased milk urea level in our sIMI groups may be the result of a mismatch between protein and energy availability for the animals (Timonen et al., 2017). In any case, the negative influence of SCC on milk urea agrees with the literature (Nyman et al., 2014). The SCC is recognized as an international standard of udder health status, with an identified cut-off of 200,000 cells/mL (Bobbo et al., 2017; Pegolo et al., 2022). Indeed, bacteria in infected

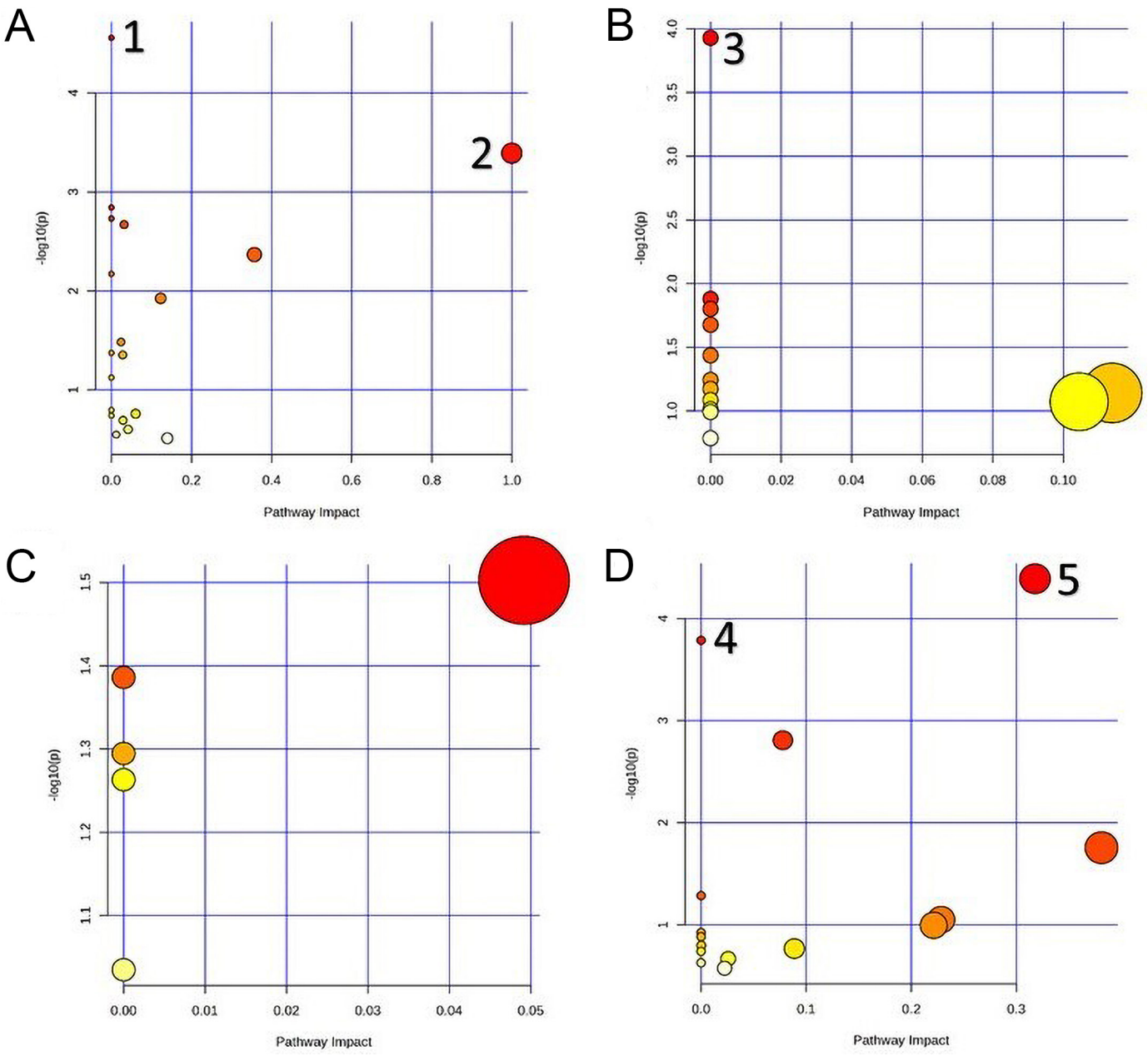

Figure 2. Bubble plot of metabolic pathways influenced by (A) first main cluster (greater concentration in control group), (B) first subcluster (greater concentration in group infected by *Prototheca* spp.), (C) second subcluster (greater concentration in group infected by *Streptococcus agalactiae*), (D) third subcluster (greater concentration in animals affected by subclinical IMI). 1: aminoacyl-tRNA biosynthesis; 2: phenylalanine, tyrosine, and tryptophan biosynthesis; 3: aminoacyl-tRNA biosynthesis; 4: aminoacyl-tRNA biosynthesis; and 5: glycine, serine, and threonine metabolism. Color gradient and symbol size represent significant metabolite changes in the corresponding pathway. Results obtained by the pathway analysis performed with MetaboAnalyst 5.0 (https://www.metaboanalyst.ca).

tissue induce an immune response that results in the flow of components, including leukocytes, from blood to milk (Åkerstedt et al., 2012; Halasa and Kirkeby, 2020). However, *Prototheca* spp. infections do not necessarily increase or change the concentration of somatic cells (Pieper et al., 2012; Wawron et al., 2013). In this study, SCC showed a value above the cut-off for mastitis identification in both sIMI-infected groups compared with control cows. In addition, although milk lactose levels did not differ among the groups, a negative relationship between SCC and milk lactose was identified, in agreement with the literature (Malek dos Reis et al., 2013; Bobbo et al., 2017; Pegolo et al., 2022).

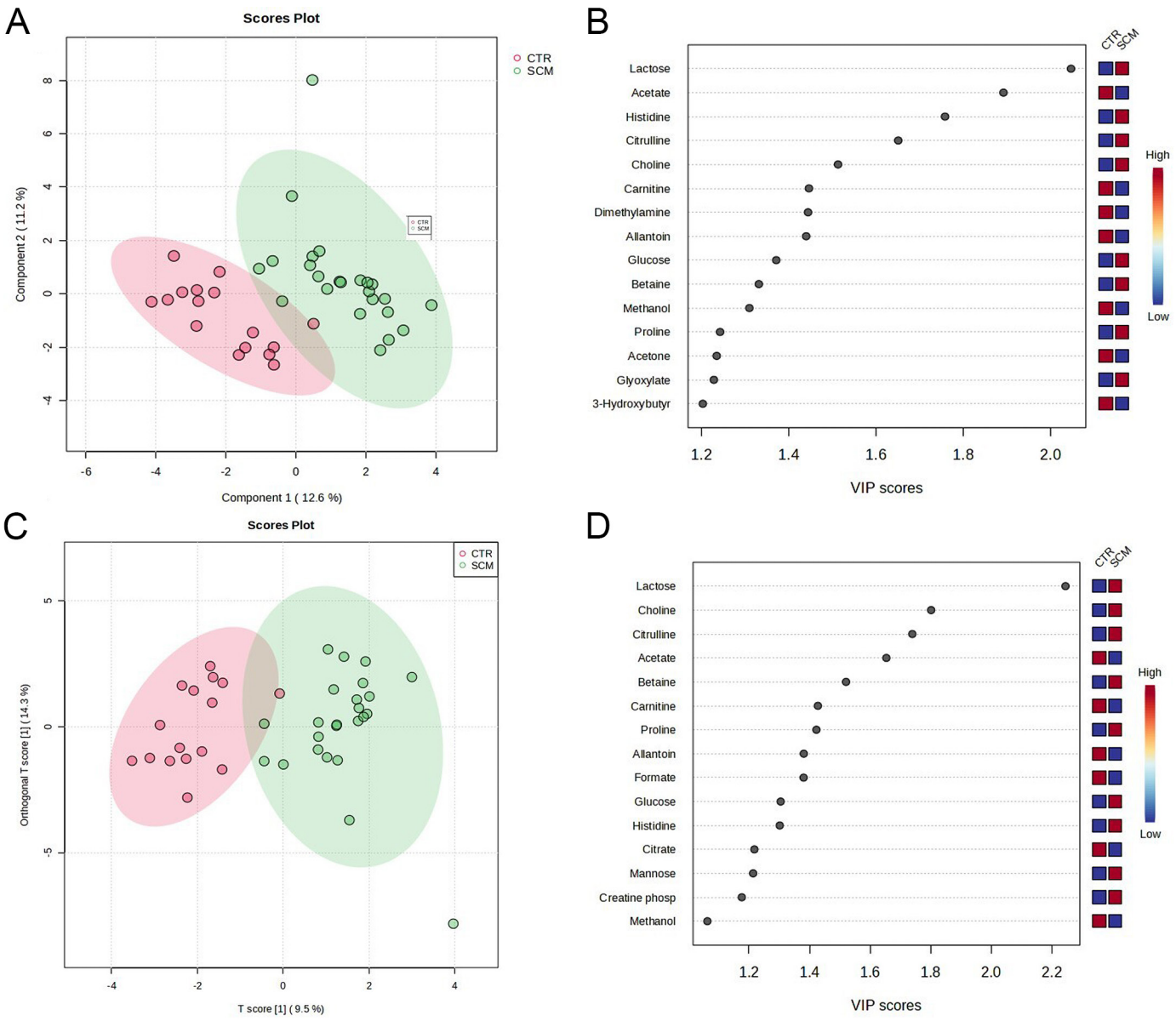

Figure 3. Score plots using animals affected by subclinical IMI versus controls (CTR; A–D) and using animals infected by *Streptococcus agalactiae* (group A) versus *Prototheca* spp. (group P; E–H). (A, E) Scores plots of partial least squares of discriminant analysis and (B, F) their variable importance in projection scores. (C, G) Orthogonal partial least squares of discriminant analysis and (D, H) their variable importance in projection scores. Results obtained by the chemometrics analysis performed with MetaboAnalyst 5.0 (https://www.metaboanalyst.ca).

The heat map of hierarchical clustering identified metabolome differences between healthy and diseased animals, as well as between SCM caused by Strep. *agalactiae* and *Prototheca* spp., which were used for pathway analysis. The sIMI groups showed a lower function of phenylalanine and tyrosine biosynthesis and protein synthesis, especially related to proteins including Phe, Ala, Ile, Thr, and Tyr. In contrast, increased synthesis of the proteins containing His, Gly, Val, and Pro was highlighted in the sIMI groups. Greater metabolism of glycine, serine and threonine has been reported in the same groups. Protein synthesis was altered between the diseased groups, with lower protein production, including the amino acids Gln, Met, and Leu, in group A compared with group P. Several alterations in amino acid metabolism and protein synthesis of SCM-affected cows have been reported in the literature using both serum (Dervishi et al., 2017; Zhang et al., 2022) and milk (Xi et al., 2017) as a matrix. However, the SCM was diagnosed only by SCC value without an investigation of the pathogen causing the mastitis in these studies. Furthermore, the

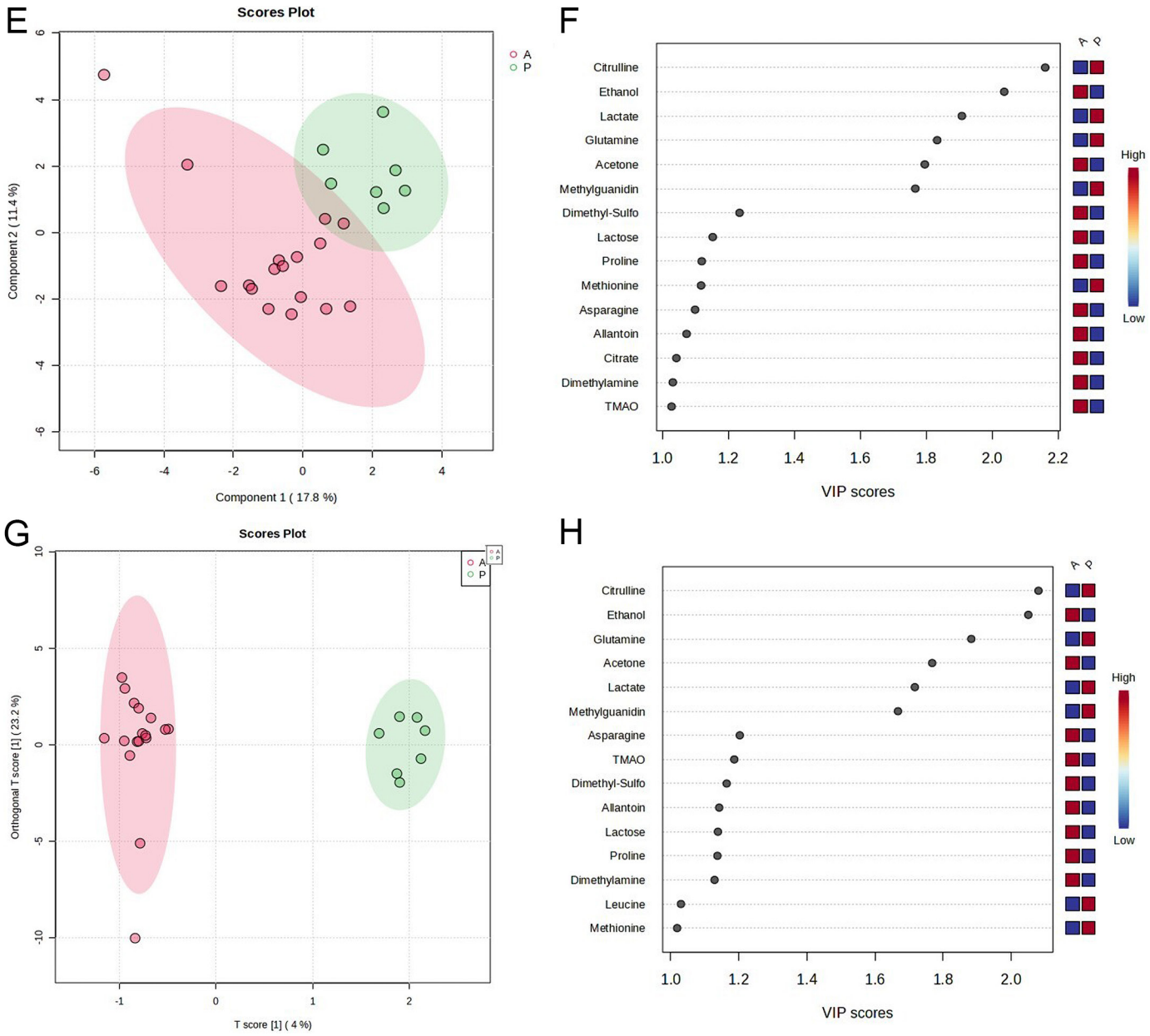

Figure 3 (Continued). Score plots using animals affected by subclinical IMI versus controls (CTR; A–D) and using animals infected by *Streptococcus agalactiae* (group A) versus *Prototheca* spp. (group P; E–H). (A, E) Scores plots of partial least squares of discriminant analysis and (B, F) their variable importance in projection scores. (C, G) Orthogonal partial least squares of discriminant analysis and (D, H) their variable importance in projection scores. Results obtained by the chemometrics analysis performed with MetaboAnalyst 5.0 (https://www.metaboanalyst.ca).

reduced level, and consequently function, of Phe and Tyr in mastitic animals may negatively affect the immune response (Hu et al., 2021).

The levels of 2 ketone bodies, BHB and acetone, and ethanol decreased in group P compared with group CTR. In addition, both groups A and P showed a decrease in acetate and dimethylamine levels. Both ethanol and dimethylamine are products of ruminal microbial metabolism, as is acetate (Kim et al., 2021; Lisuzzo et al., 2022b; Zhu et al., 2023). Their reduction may suggest impaired ruminal fermentation in animals with sIMI. Furthermore, allantoin is a purine derivate that has been proposed as a means to estimate ruminal microbial protein production (Kim et al., 2021). This metabolite was found to be reduced in group P compared with group A, indicating a potential different ruminal function among sIMI animals, possibly related to the etiological agent. In addition, ketone bodies may

Table 2. Results from ANOVA (*F*-values and *P*-values) for the representative serum metabolites expressed as μmol/L[1]

Metabolite	Group CTR (n = 16)	Group A (n = 17)	Group P (n = 7)	*F*-value	*P*-value	SCC covariate	
						F-value	*P*-value
3-Hydroxybutyrate	617.0[a]	493.0[ab]	390.0[b]	6.88	0.004	0.358	0.555
Acetate	1,616.0[a]	1,236.0[b]	1,071.0[b]	5.10	0.013	0.001	0.975
Acetone	42.3[a]	41.6[a]	24.0[b]	7.24	0.003	1.366	0.253
Allantoin	164.0[ab]	181.0[a]	135.0[b]	4.80	0.016	7.959	0.009
Asparagine	109.8[b]	91.5[a]	90.1[a]	3.87	0.034	6.336	0.018
Carnitine	147.9[a]	109.3[ab]	93.9[b]	3.67	0.039	0.273	0.605
Citrate	234.0[a]	253.0[a]	183.0[b]	3.19	0.057	0.727	0.401
Citrulline	61.1[b]	69.8[b]	100.4[a]	8.44	0.001	0.288	0.596
Dimethylamine	8.40[a]	4.34[b]	2.52[b]	2.92	0.070	0.313	0.580
Ethanol	90.7[a]	87.1[a]	37.8[b]	5.14	0.013	4.098	0.053
Histidine	193.0[b]	210.0[a]	223.0[a]	3.26	0.053	1.183	0.286
Lactose	85.9[b]	200.1[a]	163.0[a]	7.06	0.003	0.106	0.747
Methylguanidine	7.93[ab]	5.68[b]	8.62[a]	5.63	0.009	2.379	0.134
Valine	702.0[b]	833.0[a]	738.0[ab]	2.77	0.081	0.124	0.728

[a,b]Mean values in the same row that differ significantly. The significance threshold was set at $P \leq 0.05$. A *P*-value between 0.05 and 0.1 was considered as a trend.

[1]Characterization of dairy cows categorized as control (group CTR; n = 16), infected by *Streptococcus agalactiae* (group A; n = 17), and infected by *Prototheca* spp. (group P; n = 7). Results obtained by ^{1}H-NMR spectroscopy.

result from butyrate metabolism (Constable et al., 2017), which was not identified in our study. A decrease in ketone bodies may further suggest altered ruminal fermentations. However, ketone bodies can also be used as an alternative energy resource by tissues, including the mammary gland (Herdt, 2000; McArt et al., 2013). Therefore, decreased levels of ketone bodies may be partially or fully associated with a systemic metabolic change resulting from the local immune response in the mammary gland affected by sIMI (Haxhiaj et al., 2022a). Considering that both volatile fatty acids and ketone bodies can be used as energy sources in ruminants (Zhu et al., 2023), their deficiency or increased use due to sIMI may promote the onset of secondary metabolic-nutritional disorders, such as ketosis, under conditions of energy deficit in the animal (e.g., in case of negative energy balance).

The tricarboxylic acid cycle (**TCA**) is essential for energy metabolism in animals and, therefore, alterations in its functionality may have certain effects on other metabolic pathways and on the animal itself (Hu et al., 2021). Citrate is an intermediate metabolite of TCA, and its concentration was found to be reduced in the serum of mastitic dairy cows, identifying a mastitis-related alteration in energy metabolism (Haxhiaj et al., 2022a; Zhu et al., 2023). Carnitine is a metabolite responsible for the transport of fatty acids from the cytosol to the mitochondrial matrix, thereby affecting energy metabolism. A reduced concentration of carnitine was identified in milk from mastitic animals, suggesting a hindrance to energy metabolism (Xi et al., 2017; Zhu et al., 2021). In blood, carnitine has been identified as reduced 4 weeks postpartum in animals with disease (metritis, mastitis, laminitis, or retained placenta) during the transition period (Hailemariam et al., 2014). Moreover, TCA is functionally related to the urea cycle (Lisuzzo et al., 2022c). As with citrate, increased serum Cit concentrations, an intermediate of the urea cycle, have been found in mastitic animals. Allantoin and methylguanidine are also related to urea synthesis. The first metabolite may result from the degradation of uric acid (Müller et al., 2021) and is an intermediate step in urea synthesis. The second metabolite is derived from protein catabolism, particularly creatine, which is the most important nitrogen-containing compound (Chen et al., 2011). Methylguanidine has been found to be increased in humans with uremic syndrome, and some bacterial species are able to hydrolyze it (Nakajima et al., 1980; Duranton et al., 2012) resulting in urea synthesis. In this study, reduced levels of citrate, carnitine, and allantoin, and increased concentrations of Cit and methylguanidine, were observed only in group P. These findings may suggest a possible alteration of the urea cycle and synthesis and may be because of different mastitis severity or a different metabolomic pattern related to the etiologic agent causing mastitis.

The presence of bacteria and transport of cytokine from the infected udder to the systemic bloodstream were also related to increased permeability of the mammary gland, which induced a change in systemic metabolites, particularly amino acids, supporting inflammatory and immune responses (Dervishi et al., 2017; Haxhiaj et al., 2022a; Zhu et al., 2023). A possible systemic involvement of the inflammatory and immune responses can

be hypothesized in this study. Indeed, previous studies have shown that Asn levels were reduced both before and during mastitis (Haxhiaj et al., 2022a; Zhu et al., 2023). In addition, in herds with a high prevalence of mastitis, a decrease in the Asn:Asp ratio was reported, suggesting a potential relationship with the inflammatory event, which should be confirmed (Holtenius et al., 2004). Furthermore, Asn is one of the precursors of oxaloacetate in the TCA cycle (Lisuzzo et al., 2022a). In this study, Asp was not identified; however, Asn was reduced in both sIMI groups. Histidine exhibits antioxidant and anti-inflammatory qualities resulting from its ability to remove reactive oxygen species (Lisuzzo et al., 2022a). Its concentration increased in the serum of animals with SCM (Zhang et al., 2022) as in this study. The increase in His could be because of an increased level of reactive oxygen species in SCM-infected cows (Pegolo et al., 2023). Valine is one of the branched-chain amino acids whose increased concentration in blood is considered an indicator of poor metabolic health in cows (Dervishi et al., 2017; Lisuzzo et al., 2022a). Moreover, increased Val levels have been found in the blood of dairy cows before, during, and after mastitis (Dervishi et al., 2017). Branched-chain amino acids are important for the inflammatory and immune response through regulation of protein synthesis and activation of cytokine and antibody production (Li et al., 2007; Dervishi et al., 2017). Concentration of Val was increased in group A compared with group CTR.

Damage of the mammary gland membrane allows greater passage of blood constituents into the milk (Zhu et al., 2021). However, the resulting increase in the permeability of the paracellular pathway may also promote a passage of substances synthesized by lactocytes, such as lactose, to the bloodstream (Zhu et al., 2023; Pegolo et al., 2023). Indeed, increased excretion of lactose in the urine of cows and humans was associated with inflammatory states of the udder or lactating breast (Fetherston et al., 2006; Zhu et al., 2023). However, no correlation has been demonstrated at the blood level between the extent of inflammation and blood lactose concentration, although this evaluation was performed on women (Fetherston et al., 2006). In our study, an increased serum lactose level was identified in both groups with sIMI and was possibly related to the increased permeability of the damaged mammary gland.

Considering these results, the risk of energy deficit, especially during mastitis due to *Prototheca* spp., may negatively influence the immune response. This negative influence may represent a risk factor for the incidence of recurrence and chronic mastitis, and therefore further studies are needed. In addition, the energy deficit also potentially related to dysfunction of ruminal fermentations could predispose animals with subclinical mastitis to secondary metabolic disorders under particular conditions such as negative energy balance. In addition, the use of anti-inflammatories to control mastitis should be newly evaluated considering that a systemic inflammatory response is present even under mild conditions (SCM). These topics need further study, especially given that this study only considered one herd, one specific severity of mastitis (subclinical), and 2 pathogens in particular.

CONCLUSIONS

The serum metabolomic profile appears to be affected during sIMI. The changes found in this study appear to be associated with alterations in (1) ruminal fermentations, (2) energy metabolism, and (3) urea synthesis and metabolism. In addition, possible links with immune and inflammatory responses, as well as increased mammary gland permeability, were suspected. In sIMI cases, the etiological agents also seem to influence the metabolomic profile and severity of the identified alterations. Validation of these results in a larger cohort of animals is needed. Further studies comparing the metabolomic profile of blood and milk and evaluating other etiological agents are required.

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the work: AL and EF; revising the original draft: LL, AC, VB, SP, DG, RT, AB, ES, EM, CT, and MG. The authors have not stated any conflicts of interest.

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