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Integrated serum metabolomics and milk immune cell profiling reveals systemic and local responses in Holstein cows with subclinical intramammary infection

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ABSTRACT

Integrating multiple data layers could offer a better understanding of the pathogenetic mechanisms behind the development of mastitis and improve the identification of robust biomarkers for its detection. Hence, in this work we integrated serum metabolomic data from ^{1}H nuclear magnetic resonance (^{1}H-NMR) with milk leucocytes subpopulations measured with flow cytometry and milk udder health traits in healthy cows ($n = 15$, NEG) and cows with spontaneous subclinical intramammary infection (sIMI) ($n = 19$). The Data Integration Analysis for Biomarker discovery using Latent Components (DIABLO) algorithm was used to combine these datasets and identify key features for sIMI detection. The predictive ability of the selected hub variables in discriminating between NEG/sIMI animals was then assessed using receiver operating characteristic (ROC) analysis. This approach revealed a strong correlation ($r = .73$) between serum metabolomic and milk leukocytes categories. Among the most informative features selected by DIABLO we observed an increased concentration of lactose, due to the altered permeability of the mammary gland and histidine, potentially modulating immune responses. Additionally, the decreased concentrations of ruminal fermentation-associated metabolites (e.g. acetone, methanol, ethanol and 3-Hydroxybutyrate) suggest, with the onset of inflammation, a shift in energy allocation from physiological processes towards immune response. The predictive performance of the selected metabolites ranged from moderate to good. Lactose emerged as the most promising biomarker, while allantoin demonstrated high sensitivity (0.93). These findings demonstrate the potential of combining immunological and metabolomic profiling across different biofluids for mastitis detection, though larger cohort is required for testing its application.

HIGHLIGHTS

- Strong relationship highlighted between milk immune cells and serum metabolomic variables
- Moderate correlations between milk leucocytes and serum metabolomic variables, but not with specific immune cells
- Infected animals show higher lactose and histidine levels
- Infected animals have lower ruminal fermentation-associated metabolites

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Introduction

Bovine mastitis is a well-recognized inflammatory disease that continues to pose a significant challenge for the dairy sector not only as it affects animal welfare, but also hinders milk productivity and quality, resulting in substantial economic losses at the expense of the farmers (Halasa et al. 2007; Ruegg 2017). While being a complex multifactorial disease, mastitis is mainly caused by the penetration of a wide range of microorganisms into the mammary gland and can occur in either clinical or subclinical forms. While clinical mastitis is characterised by overt signs of inflammation and changes in the milk physical composition, the subclinical form is typically marked only by an increase of the somatic cell count (SCC) derived from the proliferation and migration of the leucocytes

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population in the udder (Bradley 2002). Among the two forms, subclinical mastitis represents the greater challenge, as it is 15–40 times more frequent than the clinical form (Martin et al. 2018), acts as a major unnoticed source of infection within herds and increases the risk of developing subsequent clinical mastitis, leading to higher veterinary costs and early culling (Wang et al. 2024).

In recent years, the ongoing advancement of new technologies has pushed the scientific community on a major research craze for the identification of reliable biomarker for this disease (Mbindyo et al. 2020). In this context, metabolomic approaches offer valuable insights into the phenotype of a given biofluid and the triggered metabolic pathways.^{1}H nuclear magnetic resonance (^{1}H-NMR) spectroscopy, together with mass spectrometry (MS), represents two of the most adopted techniques in metabolomic investigation. The latter generally offers higher sensitivity, but it requires complex samples pre-processing that may, in some cases, results in metabolites loss (Lisuzzo et al. 2024). By contrast, ^{1}H-NMR, presents the advantages of being non-invasive, easy to perform and highly reproducible. This analysis can be performed using either targeted or untargeted approaches. In targeted metabolomics, a predefined panel of known metabolites is quantified, focusing on specific metabolic pathways of interest, whereas untargeted metabolomics aims to comprehensively profile, without bias, a wide range of metabolites within a sample (Qiu et al. 2023). Until today, ^{1}H-NMR untargeted metabolomic studies have been successfully adopted for a more in-depth characterisation of the different metabolomic signatures between cows with mastitis and their healthy counterparts (Zhu et al. 2023), as well as identifying potential biomarkers for mastitis identification (Lisuzzo et al. 2024). Despite these advancements, focusing solely on a single layer of information may offer only a partial view of mastitis. While metabolomic data provide valuable insights, they may not fully capture the complexity of the disease. Integrating multiple layers of information and/or derived from different biological matrices can offer a more comprehensive understanding of the mechanisms underlying disease pathogenesis and improve the identification of robust biomarkers. The immune system of the mammary gland plays a crucial role in controlling inflammatory and infectious processes in dairy cows (Matteis et al. 2020); however, local immune responses are tightly interconnected with systemic immune and metabolic pathways. This is particularly important, as scientific evidence demonstrates that different mastitis-causing pathogens can elicit distinct immune responses in the mammary gland, requiring pathogen-specific immune reactions for effective protection (Thompson-Crispi et al. 2014). Even in subclinical infections, the mammary gland communicates with the systemic circulation through cytokines, metabolites and immune mediators (Ezzat Alnakip et al. 2014), leading to systemic metabolic adjustments detectable in blood. In parallel, immune cell activity in milk, particularly shifts in leukocyte subpopulations, may be influenced by systemic metabolic changes that support immune cell function, proliferation and activation (Dervishi et al. 2017). Therefore, integrating information from different biofluids (i.e. milk and blood serum) could represent a powerful approach to capture coordinated or inter-organ communication mechanisms that contribute to host-pathogen interactions.

Given this consideration, in this study we conducted an integrative analysis combining blood serum ^{1}H-NMR metabolomic traits with milk immune cells subpopulations (i.e. polymorphonuclear cells-PMN, macrophages, T-killer cells, T-helper cells and B-cells) and milk udder health traits, in Holstein cows with naturally occurring subclinical intramammary infection (sIMI) from two pathogens (*Streptococcus agalactiae* and *Prototheca* spp.) to: (i) to investigate the relationship between milk immune cells and the serum metabolomic profile of the animals and (ii) to identify putative hub variables indicative of sIMI.

Methods

Experimental design and animal field data

This study was carried out within the LATSAN project, which aimed to develop innovative tools for the evaluation of bovine udder health and the improvement of the nutritional and technological quality of milk and the EUPAHW project. Experimental procedures were approved by the Ethical Animal Care and Use Committee (OPBA- Organismo Preposto al Benessere degli Animali) of the Università Cattolica del Sacro Cuore and by the Italian Ministry of Health (protocol number: 510/2019–PR of 19/07/2019).

Holstein cows were selected from a commercial dairy herd of 450 lactating cows located in the Veneto region (Italy) regularly monitored for the presence of *S. agalactiae* and *Prototheca* spp. Herd selection was based on a prevalence study conducted by the Istituto Zooprofilattico delle Venezie (IZSVe) for the identification of the most common causative agents of mastitis in the Veneto region and also due to the ease of access on the farm location, as well as the cooperation from the dairy farm operators.

A first bacteriological screening (time 0, T0) was conducted on a cohort of 188 multiparous, mid-lactating cows to identify negative animals and those with subclinical infection caused by *S. agalactiae* or *Prototheca* spp. These animals underwent a second bacteriological testing at T1, two weeks after T0 and between the two sampling times the animals were regularly monitored by the farmer and local veterinarian, to ensure they maintained the subclinical condition of mastitis. Animals were classified as negative only if all four mammary quarters tested negative, whereas positivity was assigned when at least one quarter was positive for either pathogen. Based on the results of T0 and T1, three groups were defined: (i) cows negative at both time points and with no history of subclinical mastitis ($n = 15$) and (ii) positive at the bacteriological examination and with subclinical mastitis from either *S. agalactiae* ($n = 10$) or *Prototheca* spp. ($n = 9$). Moreover, the attribution of the negativity was made only if the four mammary quarters tested negative, on the other hand positivity was attributed if at least one quarter was positive for either one of the two pathogens. Moreover, animals with co-infections, clinical signs of mastitis and treated with antibiotics were excluded from the trial. All the details on animals and experimental plan are reported by Bisutti et al. (2023).

Microbiological analysis on milk samples

Microbiological analysis of milk samples was conducted by the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe) laboratory (Legnaro, PD, Italy). Refrigerated samples at 4 °C were received within 4 h of collection, frozen and analysed within three days. Pegolo et al. (2022b) reported the specifics of the analysis. Briefly, 10 µL of every composite sample were inoculated in each of the following selective media: (i) thallium kristalviolette tossin agar (TKT; IZSVe internal production) and (ii) *Prothoteca* isolation medium (PIM; IZSVe internal production). Suspected colonies of *S. agalactiae* were confirmed using the Christie–Atkins–Munch- Peterson test (NMC, 2017) after 24 h of incubation. At the same time, *Prototheca* isolation medium plates were observed at 24, 48 and 72 h, and the wet mount method confirmed suspected colonies. In addition, a screening of the most common microorganisms potentially causative for mastitis such as *Staphylococcus aureus* and *Streptococcus uberis*, as well as some environmental ones, such as *Streptococcus* spp., *Staphylococcus* spp., *Klebsiella* spp. and *Enterococcus* spp. was conducted to avoid possible bias in the trial. Samples testing positive for the presence of environmental microorganisms were excluded from the subsequent analyses.

Milk composition, udder health traits and flow cytometry analysis

Individual milk samples were collected from each cow. A first aliquot underwent milk composition (protein, casein, lactose, fat and urea content), as well as some quality traits as pH and conductivity (mS/cm) were carried out through the FT6000 infra-red analyser (Foss A/S, Hillerød, Denmark) on fresh samples, while SCC and differential somatic cell count (DSCC) were measured through the Fossomatic 7 DC analyser (Foss A/S).

An additional 50-mL aliquot from each sample was immediately transferred to the cell laboratory of the Comparative Biomedicine Department (BCA) of the University of Padova (Italy) for the determination of milk immune cell populations through flow cytometry. All analyses were carried out within 12 h upon collection with milk always stored at 4 °C. The detailed flow cytometry methodology and analysis are reported by Pegolo et al. (2022b) and Bisutti et al. (2023). Flow cytometric analyses were performed using a CyFlow Space flow cytometer (Sysmex Partec GmbH, Norderstedt, Germany) fitted with a blue laser (488 nm), a red laser (635 nm) and a UV laser. The data were analysed with the FlowMax software version 2.82 (Sysmex Partec GmbH, Norderstedt, Germany).

Identification of blood serum metabolites

The detailed specifics of the untargeted metabolomic analysis are reported by Lisuzzo et al. (2024). Briefly, ^{1}H-NMR spectra were acquired at 298 K using an AVANCE III spectrometer (Bruker, Milan, Italy) operating at a frequency of 600.13 MHz and equipped with Topspin 3.5 software. To suppress broad resonances from large molecules, a Carr-Purcell-Meiboom-Gill (CPMG) filter was applied, consisting of 400 echoes with a τ of 400 µs and a 180° pulse duration of 24 µs, resulting in a total filter duration of 330 ms, as described by Zhu et al. (2020). Residual water signals were suppressed using a presaturation technique, then 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). Signal attribution was performed in an untargeted manner by comparing the chemical shift (position along the spectrum) and multiplicity (signal shape) using Chenomx

software (version 8.3, Chenomx Inc., Edmonton, AB, Canada). This was done by referencing the Chenomx (version 10, 336 compounds) and HMDB (version 2, 643 compounds) databases. The process involved overlaying the acquired spectra with those of pure compounds from the databases. Molecule quantification was conducted using rectangular integration, selecting a signal that was free from interferences.

Statistical analysis

The integrative approach was conducted using the DIABLO (Data Integration Analysis for Biomarker discovery using Latent Components) algorithm of the mixOmics R package (http://mixomics. org/, v. 6.18.1;Rohart et al. 2017). Prior to the integration analysis all the variables of all the data sets were centred and scaled using the algorithm default parameter of mean 0 and variance 1. The evaluation of the relationships among the data sets was evaluated by adding a categorical variable (i.e. the infection status of the animals). A first attempt to evaluate the pathogen-specific signatures among data set was made, but no significant results were highlighted. Therefore, healthy cows ($n = 15$) were compared to infected individuals ($n = 19$). DIABLO seeks to estimate latent components by modelling and maximising the correlation between pairs of pre-specified datasets to unravel similar functional relationships (Singh et al. 2019). In specific, host-related traits included serum metabolomic profile ($n = 42$) measured with ^{1}H-NMR (Met); milk leucocytes subpopulation measured with flow cytometry ($n = 6$; immune system- IS); milk udder health (UH) traits ($n = 6$); and cows' parity and DIM (Host). The number of components was set as 1 (one less than the number of experimental groups, which were 2), which is sufficient to achieve the best discriminative performance according to the algorithm developers' recommendation (Rohart et al. 2017). The model was first fine-tuned using leave-one-out cross-validation by splitting the data into training and testing. Then, classification error rates were calculated using balanced error rates (BERs) between the predicted variables and the class labels' centroid. Only interactions with $|r| \geq .56$ were visualised using CIRCOS.

The top 20 most informative metabolomic variables identified by the DIABLO algorithm were then submitted to the MetaboAnalyst 5.0 software (https://www.metaboanalyst.ca). A quantitative enrichment analysis was then performed to assess influenced pathways based on the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database.

Validation of hub metabolomic variables

To validate the abovementioned hub variables as putative markers for mastitis infection, we performed the receiver operating characteristics (ROC) and precision-recall analyses using the R package pROC (v. 1.18.0) package to quantify the infection status predictive power of hallmark variables. The area under the curve (AUC), which is the plot of sensitivity and 1-specificity, was calculated to check the accuracy of the markers in distinguishing between infected and healthy cows.

Results

Integrative analysis

Figure 1(A) displays the correlation plot among the different datasets. Using the DIABLO approach, we observed the strongest covariation between Met and IS categories ($r = .73$), followed by milk udder health variables (UH) and immune cells subpopulation ($r = .63$) and metabolomic traits and milk udder health ($r = .56$). As expected, no significant covariation was observed when considering the relationships with the Host category (i.e. DIM and parity) and all the other sets of variables ($r = .02$, .05 and .16, for Host-IS, Host-UH and Host-Met, respectively). Despite the strong relationships evidenced, we observed only a partial clusterisation of the samples according to the infection status and no clear separation between animals positive for *S. agalactiae* and *Prototheca* spp. (Figure 1(A)). For this reason, all downstream analyses were conducted by comparing healthy and sIMI animals, regardless of the pathogen.

Then, to better investigate the biological meaning of the predicted model, we investigated the behaviour of the DIABLO-selected features having the highest co-variation in respect to the infection status of the animals. Figure 1(B) reports the top 20 metabolites that were selected by the algorithm as the most informative ones for the cows' infection status. The first latent variable of the Met dataset highlighted higher levels of Lactose and Histidine (His) in cows with sIMI, while dimethylamine, acetone, methanol, allantoin and ethanol increased in healthy animals. Other potentially informative metabolites associated with the presence of infection were betaine, choline and valine. Concerning the IS category, DIABLO results supported the activation of the immune system response in cows with mastitis, with the proliferation of total leucocytes, more specifically polymorphonuclear and T-killer cells and to less extent B-cells,

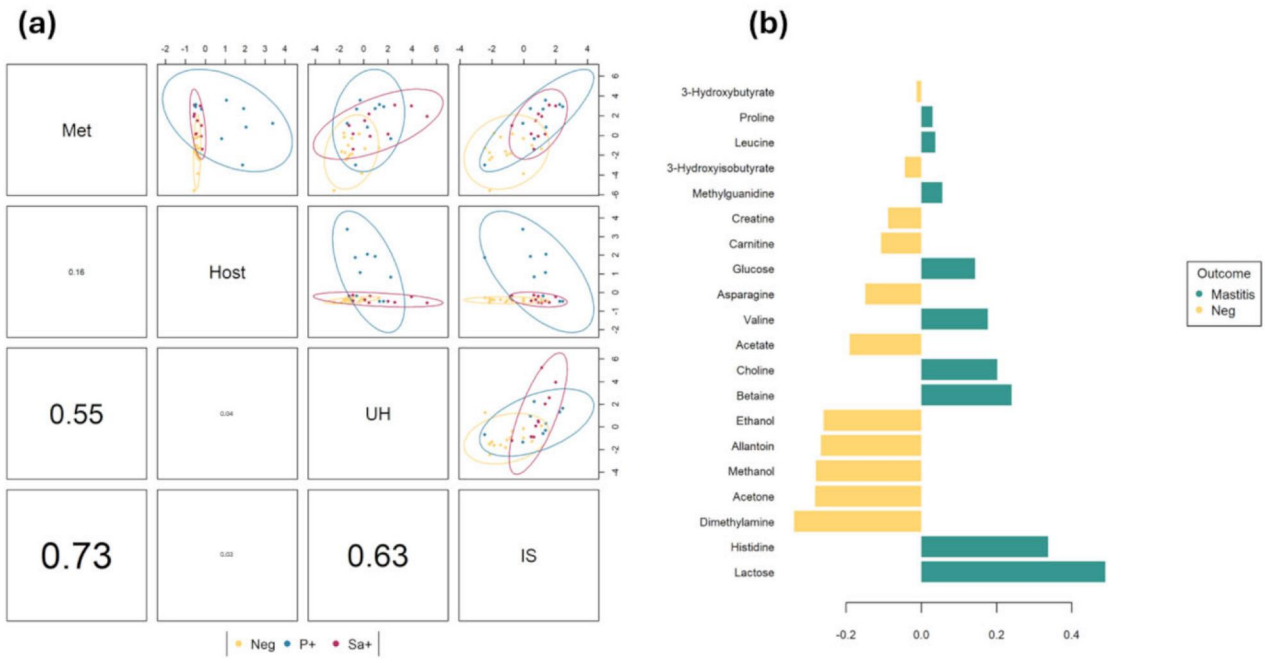

Figure 1. Correlation plot among the different sets of categories (a); the loading plot of the top 20 ^{1}H-NMR metabolomic variables identified by the DIABLO algorithm (b). The negative values of the loading weights (yellow bars) indicate that the corresponding variables had higher values in healthy animals. The positive loading value (green bars) signify that the corresponding variables had increased concentration in infected animals. IS: immune system; UH: udder health; MET: ^{1}H-NMR blood serum metabolomic variables; P+: positive to *Prototheca* spp.; Sa+: positive to *S. agalactiae*.

T-helper cells and macrophages (Supplementary Figure S1). Moreover, concerning the UH category, we confirmed DSCC, SCC and the casein index as the most informative variables selected by the algorithm for the infection status of the animals, with DSCC and SCC increasing with the presence of sIMI and casein index decreasing (Supplementary Figure S1).

Finally, the CIRCOS plot (Figure 2) allowed us to better investigate the correlation existing among the different traits within each dataset. Total leucocytes were found negatively correlated with most of the serum metabolic traits and particularly with methanol ($r = -.72$), dimethylamine ($r = -.69$), acetate ($r = -.69$), acetone ($r = -.67$). Conversely, total leucocytes were positively correlated with serum lactose ($r = .70$) and histidine ($r = .57$). Among milk UH traits casein index and lactose were positively correlated with dimethylamine ($r = .57$ and $.60$), acetone ($r = .56$ and $.59$) and acetate ($r = .58$ and $.60$), while milk conductivity was negatively correlated with methanol ($r = -.60$), dimethylamine ($r = -.63$) acetone ($r = -.62$) and acetate ($r = -.63$).

The 20 metabolites identified with the algorithm were used to identify potentially informative metabolic pathways which could be affected by the health status of the mammary gland. Lactose and glucose were included in the galactose metabolism (Holm p value $= .0013$), while His fell within both the histidine (Holm p value $= .0509$) and beta-alanine metabolism (Holm p value $= .0509$).

Validation of metabolomic putative hub variables

As a first attempt of validating the potentially informative metabolomic biomarkers identified with the DIABLO algorithm we performed a ROC analysis to assess the predictive ability of those traits. Table 1 reports the detailed predictive ability of the top 10 hub metabolomic variables identified with the DIABLO algorithm. All traits displayed overall moderate to good prediction performances in terms of discriminating healthy and mastitis animals, with sensitivity > 0.60, specificity > 0.53, accuracy > 0.68 and precision > 0.61.

Discussion

Even if subclinical mastitis is a well-established issue, and there have been several steps forward in getting to know it better, the ultimate challenge is to identify reliable biomarkers for its early detection. This study provides a first integrative perspective on subclinical intramammary infection by combining serum metabolomics, milk immune cell populations and udder health traits using the DIABLO framework. Rather than focusing on single biomarkers, this approach identifies

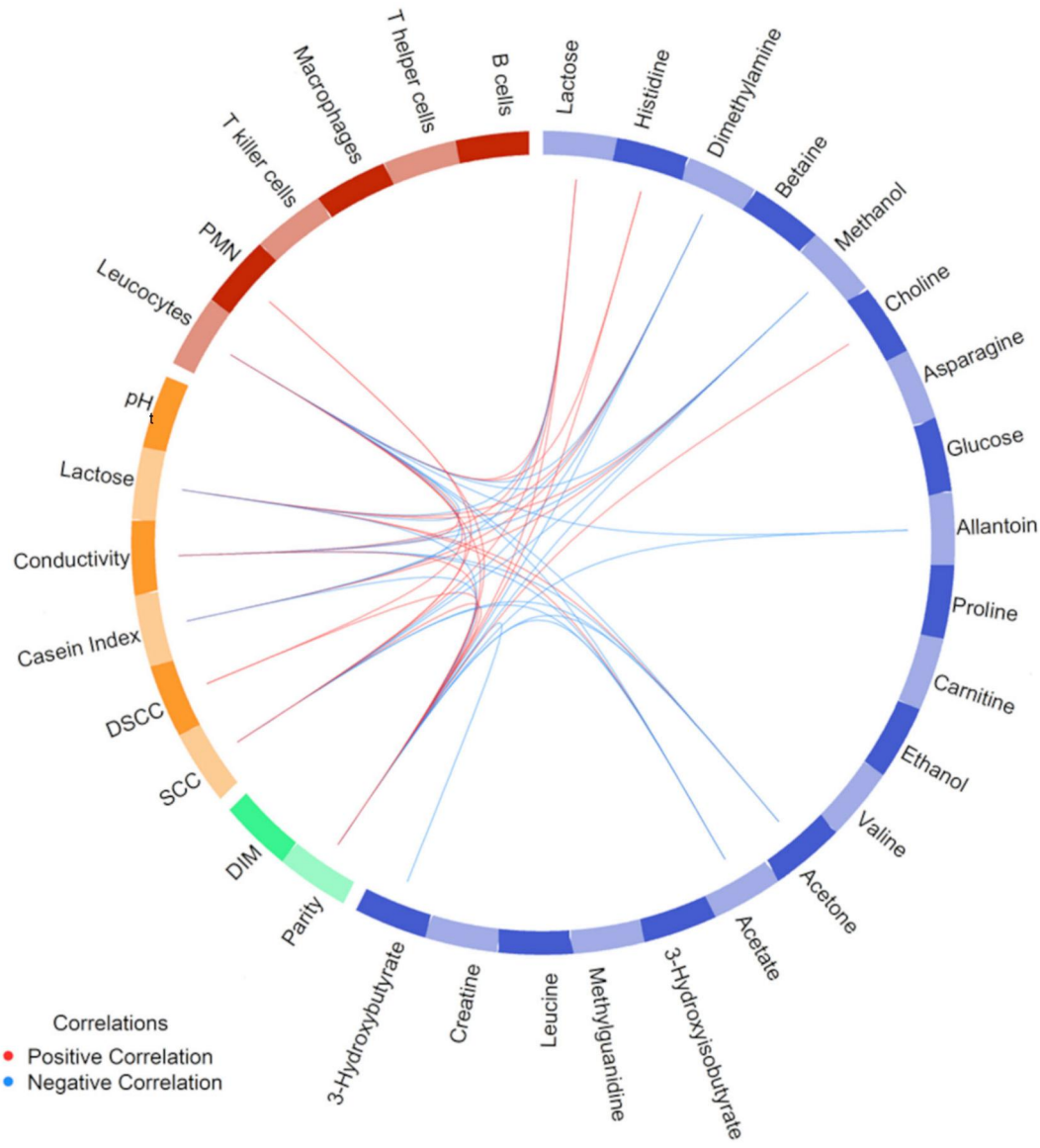

Figure 2. CIRCOS plot showing the correlation among the candidate variables of each dataset.

interconnections across multiple biological layers, revealing coordinated changes between systemic metabolism and local immune activity in the mammary gland. Importantly, by integrating data from different biological matrices (blood and milk) it was possible to capture cross-compartment interactions, demonstrating that systemic metabolic changes are reflected in local immune responses. The strong correlation between overall serum metabolomic variables (MET) and total milk leukocytes (IS; $r = .73$) categories underscores the potential of multi-omics integration to uncover functional relationships that are not evident when analysing individual datasets separately.

Sources of variation potentially affecting the evaluated traits were minimised by excluding primiparous cows and animals during their periparturient period (Bisutti et al. 2023). Indeed, in Figure 1(A), the Host category (i.e. animals' parity and DIM) poorly covaried with all the other variables categories.

Table 1. Predictive performances of the hub variables identified with DIABLO.

	AUC	Specificity	Sensitivity	Accuracy	Precision
Acetate	0.67	0.53	0.93	0.71	0.61
Acetone	0.72	0.74	0.67	0.71	0.67
Allantoin	0.74	0.53	0.93	0.71	0.61
Betaine	0.78	0.89	0.60	0.76	0.82
Dimethylamine	0.87	0.95	0.67	0.82	0.91
Ethanol	0.68	0.68	0.67	0.68	0.63
Histidine	0.79	0.84	0.67	0.76	0.77
Lactose	0.91	0.74	1.00	0.85	0.75
Methanol	0.79	0.74	0.80	0.76	0.71
Choline	0.63	0.37	1.00	0.65	0.56

Note: AUC: area under the curve.

Among the candidate serum metabolites identified by DIABLO, it was observed that lactose and histidine were the variables having the highest loading scores

and that increased in animals with sIMI. Lactose is one of the most important components of milk. Several scientific works have observed a decreased concentration of this carbohydrate in milk in cows having both clinical and subclinical mastitis (Antanaitis et al. 2021; Pegolo et al. 2022a). Decrease in milk lactose might be attributable both to the fact that it represents one of the most important energy substrates for many bacteria, including *Streptococcus* (Thomas et al. 2016), but also to the increase in the permeability of the mammary gland related to tight junction impairment (Televičius et al. 2021), which could result in a leakage of this carbohydrate into the bloodstream. These findings confirm that the quantification of lactose in both milk and blood provides a reliable indicator of altered permeability in the mammary gland, which might underline the presence of mastitis. Histidine, which also displayed an increased concentration in infected animals, is an essential amino acid which has been associated with an anti-inflammatory role in various animal species (Moro et al. 2020). Specifically, its antioxidant and anti-inflammatory qualities seem to be related to its ability to suppress the production of pro-inflammatory cytokines (e.g. interleukin 6 and tumour necrosis factor alpha) in macrophages stimulated by lipopolysaccharides (Andou et al. 2009). Moreover, His may also enhance the activities of antioxidant enzymes such as glutathione peroxidase in plasma and superoxide dismutase in red blood cells (Kopeć et al. 2013). Furthermore, the antioxidant and anti-inflammatory ability seemed to be linked to the ability of His to remove reactive oxygen species (ROS) (Lisuzzo et al. 2022a). Previous studies reported an increased concentration of His in milk (Luangwilai et al. 2021), urine (Zwierzchowski et al. 2024) and serum (Zhang et al. 2022), in accordance to what observed in this study. This finding might be attributable to the increase levels of ROS in cows with sIMI, as reported by Pegolo et al. (2023). Notably, histidine was also included in ß-alanine metabolism pathway. ß-alanine is a non-essential amino acid that works by combining histidine to generate carnosine which was first reported to effectively scavenge reactive carbonyl species (RCS) in a mouse model of diabetes (Albrecht et al. 2017), therefore potentially included in the scavenging activity for removing reactive species. Furthermore, evidence has reported that cows in negative energy balance, or other stress conditions, can mobilise amino acids from muscle proteins, leading to an increased plasma concentration of 3-methylistidine, a product of the post-translational methylation of histidine (Houweling et al. 2012). Although no direct association with mastitis has been reported to date, elevated histidine concentrations may therefore also reflect mild muscle catabolism driven by increased systemic stress responses, which is consistent with an active inflammatory state. Taken together, the positive correlation ($r = .57$) observed between total leucocytes and histidine further strengthens its involvement in the modulation of the inflammation process.

Other metabolites, such as choline and betaine, were also identified as hub variables with elevated levels in infected animals. Choline is a quaternary amine and essential nutrient that participates in different cellular processes, including the synthesis of different phospholipids, that are important for cell membrane biogenesis and fundamental in cell differentiation and phagocytosis (Ghorbani et al. 2023). Recent evidence has started to highlight the potential role of choline in the regulation of macrophages activation (Snider et al. 2018). In the context of mastitis, increased concentration of choline was reported by Bobbo et al. (2022) in dairy cows with high SCC, in accordance with the results of this study. Betaine, also known as trimethylglycine, is a compound involved in choline-mediated one carbon metabolism, cell membrane integrity, signal transduction (Xia et al. 2018) and, moreover, is an essential osmoprotectant (Zhao G et al. 2018). Recent studies have highlighted its anti-inflammatory potential, especially through modulation of the nuclear factor-κB (NF-κB) and nuclear factor erythroid 2-related factor 2 (Nrf2) signalling pathway (Zhao N et al. 2022), which could explain its increased levels in infected animals in the present study. Furthermore, these two metabolites were positively correlated with total leucocytes, further supporting their potential role in immune modulation.

Among the most important candidate metabolomic variables identified by DIABLO several metabolites decreased concentrations in infected animals, including dimethylamine, acetone, methanol, allantoin, ethanol and 3-Hydroxybutyrate (BHB). Interestingly, the concentration of BHB and acetone was lower in sIMI animals. A decrease in BHB might be attributable to different factors such as increased levels of blood glucose (Figure 1), reduced rumen motility and an impairment of hepatic ketogenesis (Moyes et al. 2014). Other studies have highlighted and increase in these two ketone bodies (Dervishi et al. 2017; Stanojević et al. 2023), especially in periparturient mastitis cows. Indeed, this finding is probably linked to the fact that early lactating cows often experience a negative energy balance (NEB) condition due to their decreased

feed intake and increased energy demand, which leads to an increased risk of developing mastitis, as a consequence of immunosuppression due to NEB. The decreased concentration of dimethylamine, ethanol, methanol and allantoin could be again linked to a reduced ruminal fermentation and changes in methane emission in animals with sIMI in respect to healthy ones. Indeed, ethanol, methanol and dimethylamine are products of ruminal microbial metabolism (Lisuzzo et al. 2022b; Fiore et al. 2023; Zhu et al. 2023), while allantoin, a purine derivative, can be used as an indicator for recovery of microbial proteins at the duodenal level (Deshpande et al. 2012). Interestingly, all these metabolites were negatively correlated with milk leucocytes, therefore highlighting a switch in the physiological and metabolic processes with the onset of inflammation.

Overall, moderate to high correlations were observed only between total leucocytes and serum metabolites, while no significant correlations were observed when considering individual immune cells subpopulations (e.g. PMN, macrophages, T-cells). This outcome might be attributable both to the subclinical type of infection, but also to the fact that we integrated information from two different matrices (i.e. milk and serum) which might have led to a reduced impact on this relationship. This might also explain how, unexpectedly, no differences were observed in the distribution of serum metabolites in animals with subclinical mastitis caused by *S. agalactiae* or *Prototheca* spp., despite the significant differences in milk leucocytes distributions (Pegolo et al. 2022b). Moreover, it could be hypothesised that systemic metabolic adaptations may reflect a generalised early immune response rather than one specific to the infecting pathogen. This finding points towards universal metabolic biomarkers for subclinical mastitis, which could simplify monitoring strategies across herds exposed to multiple pathogens. One limitation of the present study that may partially explain the weak correlations among the metabolomic variables and milk individual immune cell populations, is that animals were sampled at a single time point. As a consequence, no information was available on the stage or progression of infection, which may influence both immune and metabolic profiles. Moreover, local immune responses in the mammary gland and systemic metabolic alterations may occur with different temporal dynamics, potentially introducing a delay between changes detectable in milk and those measurable in blood. This limitation is particularly relevant in the context of subclinical mastitis, a condition characterised by mild and often localised inflammatory responses, which may not elicit strong systemic metabolic alterations. This warrants the need before future longitudinal studies aimed at monitoring changes in the metabolome and immune-related variables over time. Moreover, to strengthen and validate the results obtained in this work, it would be fundamental to increase the sample size of the investigated animals.

Among the hub metabolites, the ROC analysis highlighted lactose having the highest predictive performance for discriminating sIMI cows (AUC = 0.91), further reinforcing its essential role in mastitis detection. Also, allantoin displayed promising prediction performances, especially in terms of sensitivity (= 0.93). As this metabolic product is often the result of the combination of uric acid with ROS species (Lalwani et al. 2015), it would be interesting to further explore its role as a biomarker for cell damage or inflammation process. While these results could be promising, their predictive performance was assessed only using database of the current study. Therefore, external validation in independent and larger cohorts will be necessary to confirm the robustness and generalisability of the findings. Finally, the integration of systemic data with milk metabolomic profiles will be fundamental to provide a more detailed picture of mastitis pathogenesis and strengthened the identification of candidate markers.

Conclusions

This work provides a first integrative analysis of milk immune cells subpopulations and serum metabolomic traits upon sIMI from *S. agalactiae* and *Prototheca* spp. in dairy cattle. Interestingly, we observed a significant correlation between the immunological and metabolomic variables, even though they derive from two different matrices. Serum metabolomic variables showed a moderate correlation with milk total leucocytes, but not with the individual immune cell populations, likely reflecting the mild and localised nature of subclinical infection.

Among the putative most informative variables selected by the algorithm, lactose and histidine were the most significant ones, displaying an increase in sIMI animals highlighting, for the latter variable, a role in the modulation in the immune response. Moreover, the observed decrease in metabolites involved in ruminal fermentation might suggest that, with the onset of inflammation, the energy needed for physiological processes could be directed for sustaining the immune response. Finally, the ROC analysis further

confirmed the role of lactose as a powerful biomarker for mastitis detection and proposed allantoin as a potential trait to be further investigated.

Overall, these findings demonstrate the potential of combining immunological and metabolomic profiling across different biofluids to capture early and subtle systemic signatures of subclinical intramammary infections in dairy cattle. Such multi-biofluid approaches are particularly relevant for subclinical conditions, where single biomarkers may lack sufficient sensitivity and may therefore support the development of precision monitoring tools in dairy farms. Future studies on larger and longitudinally monitored cohorts will be essential to validate these results and strengthen their translational applicability.

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Author contributions

SP conceived the study. VB participated in the sampling activity. AL and EF performed metabolomic analysis and data interpretation. MEG helped with flow cytometry analysis. AC contributed to experimental design. VB performed the integration analysis. VB, SP and DG contributed to data interpretation. VB wrote the initial draft. All authors reviewed and approved the final draft.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

References

Albrecht T et al. 2017. Carnosine attenuates the development of both type 2 diabetes and diabetic nephropathy in BTBR Ob/Ob mice. Sci Rep. 7(1):44492. https://doi.org/10.1038/srep44492

Andou A et al. 2009. Dietary histidine ameliorates murine colitis by inhibition of proinflammatory cytokine production from macrophages. Gastroenterology. 136(2):564.e2–574.e2. https://doi.org/10.1053/j.gastro.2008.09.062

Antanaitis R, Juozaitienė V, Jonike V, Baumgartner W, Paulauskas A. 2021. Milk lactose as a biomarker of subclinical mastitis in dairy cows. Animals. 11(6):1736. https://doi.org/10.3390/ani11061736

Bisutti V et al. 2023. Transcriptome - wide mapping of milk somatic cells upon subclinical mastitis infection in dairy cattle. J Anim Sci Biotechnol. 14(1):93. https://doi.org/10.1186/s40104-023-00890-9

Bobbo T et al. 2022. Nuclear magnetic resonance spectroscopy to investigate the association between milk metabolites and udder quarter health status in dairy cows. J Dairy Sci. 105(1):535–548. https://doi.org/10.3168/jds.2021-20906

Bradley AJ. 2002. Bovine mastitis: an evolving disease. Vet J. 164(2):116–128. https://doi.org/10.1053/tvjl.2002.0724

Dervishi E et al. 2017. GC-MS metabolomics identifies metabolite alterations that precede subclinical mastitis in the blood of transition dairy cows. J Proteome Res. 16(2):433–446. https://doi.org/10.1021/acs.jproteome.6b00538

Deshpande KY, Mehra UR, Singh P, Verma AK. 2012. Milk allantoin content as an indicator to assess rumen microbial protein synthesis. Anim Nutr Feed Technol. 12(2):229–239.

Ezzat Alnakip M et al. 2014. The immunology of mammary gland of dairy ruminants between healthy and inflammatory conditions. J Vet Med. 2014:659801–659831. https://doi.org/10.1155/2014/659801

Fiore E et al. 2023. Serum metabolomics assessment of etiological processes predisposing ketosis in water buffalo during early lactation. J Dairy Sci. 106(5):3465–3476. https://doi.org/10.3168/jds.2022-22209

Ghorbani P et al. 2023. Choline metabolism underpins macrophage IL-4 polarization and RELMα up-regulation in helminth infection. PLoS Pathog. 19(9):e1011658. https://doi.org/10.1371/journal.ppat.1011658

Halasa T, Huijps K, Østerås O, Hogeveen H. 2007. Economic effects of bovine mastitis and mastitis management: a review. Vet Q. 29(1):18–31. https://doi.org/10.1080/01652176.2007.9695224

Houweling M, Van Der Drift SGA, Jorritsma R, Tielens AGM. 2012. Technical note: quantification of plasma 1- and 3-methylhistidine in dairy cows by high-performance liquid chromatography – tandem mass spectrometry. J Dairy Sci. 95(6):3125–3130. https://doi.org/10.3168/jds.2011-4769

Kneen MA, Annegarn HJ. 1996. Algorithm for fitting XRF, SEM and PIXE X-ray spectra backgrounds. Nucl Instrum Methods Phys Res Sect B Beam Interact Mater Atoms. 109-110:209–213. https://doi.org/10.1016/0168-583X(95)00908-6

Kopeć W et al. 2013. Influence of different histidine sources and zinc supplementation of broiler diets on dipeptide content and antioxidant status of blood and meat. Br

Poult Sci. 54(4):454–465. https://doi.org/10.1080/00071668.2013.793295
Lalwani P et al. 2015. Serum thiols as a biomarker of disease activity in lupus nephritis. PLoS One. 10(3):e0119947. https://doi.org/10.1371/journal.pone.0119947
Liland KH, Almøy T, Mevik BH. 2010. Optimal choice of baseline correction for multivariate calibration of spectra. Appl Spectrosc. 64(9):1007–1016. https://doi.org/10.1366/000370210792434350
Lisuzzo A et al. 2024. Serum metabolome differences associated with subclinical intramammary infection caused by *Streptococcus agalactiae* and *Prototheca* spp. in multiparous dairy cows. J Dairy Sci. 107(3):1656–1668. https://doi.org/10.3168/jds.2023-23851
Lisuzzo A et al. 2022a. Changes in plasma fatty acids profile in hyperketonemic ewes during early lactation: a preliminary study. Sci Rep. 12(1):17017. https://doi.org/10.1038/s41598-022-21088-5
Lisuzzo A et al. 2022b. Differences in the serum metabolome profile of dairy cows according to the BHB concentration revealed by proton nuclear magnetic resonance spectroscopy (1H-NMR). Sci Rep. 12(1):2525. https://doi.org/10.1038/s41598-022-06507-x
Luangwilai M, Duangmal K, Chantaprasarn N, Settachaimongkon S. 2021. Comparative metabolite profiling of raw milk from subclinical and clinical mastitis cows using 1H-NMR combined with chemometric analysis. Int J Food Sci Tech. 56(1):493–503. https://doi.org/10.1111/ijfs.14665
Martin P, Barkema HW, Brito LF, Narayana SG, Miglior F. 2018. Symposium review: novel strategies to genetically improve mastitis resistance in dairy cattle. J Dairy Sci. 101(3):2724–2736. https://doi.org/10.3168/jds.2017-13554
Matteis GD et al. 2020. Flow cytometry-detected immunological markers and on farm recorded parameters in composite cow milk as related to udder health status. Vet Sci. 7(3):1–10. https://doi.org/10.3390/VETSCI7030114
Mbindyo CM, Gitao GC, Mulei CM. 2020. Prevalence, etiology, and risk factors of mastitis in dairy cattle in Embu and Kajiado counties, Kenya. Vet Med Int. 2020:8831172–8831112. https://doi.org/10.1155/2020/8831172
Moro J, Tomé D, Schmidely P, Demersay TC, Azzout-Marniche D. 2020. Histidine: a systematic review on metabolism and physiological effects in human and different animal species. Nutrients. 12(5):1414. https://doi.org/10.3390/nu12051414
Moyes KM, Larsen T, Sørensen P, Ingvartsen KL. 2014. Changes in various metabolic parameters in blood and milk during experimental *Escherichia coli* mastitis for primiparous Holstein dairy cows during early lactation. J Anim Sci Biotechnol. 5(1):47. https://doi.org/10.1186/2049-1891-5-47
National Mastitis Council. 2017. Laboratory handbook on bovine mastitis. 3rd ed. National Mastitis Council, Inc.
Pegolo S et al. 2023. Blood biochemical changes upon subclinical intramammary infection and inflammation in Holstein cattle. J Dairy Sci. 106(9):6539–6550. https://doi.org/10.3168/jds.2022-23155
Pegolo S et al. 2022a. Quarter-level analyses of the associations among subclinical intramammary infection and milk quality, udder health, and cheesemaking traits in Holstein cows. J Dairy Sci. 105(4):3490–3507. https://doi.org/10.3168/jds.2021-21267
Pegolo S et al. 2022b. *Streptococcus agalactiae* and *Prototheca* spp. induce different mammary gland leukocyte responses in Holstein cows. JDS Commun. 3(4):270–274. https://doi.org/10.3168/jdsc.2022-0216
Qiu S et al. 2023. Small molecule metabolites: discovery of biomarkers and therapeutic targets. Signal Transduct Target Ther. 8(1):132. https://doi.org/10.1038/s41392-023-01399-3
Rohart F, Gautier B, Singh A, Lê Cao KA. 2017. MixOmics: an R package for 'omics feature selection and multiple data integration. PLoS Comput Biol. 13(11):e1005752. https://doi.org/10.1371/journal.pcbi.1005752
Ruegg PL. 2017. A 100-year review: mastitis detection, management, and prevention. J Dairy Sci. 100(12):10381–10397. https://doi.org/10.3168/jds.2017-13023
Singh A et al. 2019. DIABLO: an integrative approach for identifying key molecular drivers from multi-omics assays. Bioinformatics. 35(17):3055–3062. https://doi.org/10.1093/bioinformatics/bty1054
Snider SA et al. 2018. Choline transport links macrophage phospholipid metabolism and inflammation. J Biol Chem. 293(29):11600–11611. https://doi.org/10.1074/jbc.RA118.003180
Stanojević J et al. 2023. Assessment of mastitis patterns in Serbian dairy cows: blood serum metabolic profile and milk composition parameters. Pathogens. 12(11):1349. https://doi.org/10.3390/pathogens12111349
Televičius M et al. 2021. Inline milk lactose concentration as biomarker of the health status and reproductive success in dairy cows. Agriculture. 11(1):38. https://doi.org/10.3390/agriculture11010038
Thomas FC et al. 2016. Mastitomics, the integrated omics of bovine milk in an experimental model of: *Streptococcus uberis* mastitis: 3. untargeted metabolomics. Mol Biosyst. 12(9):2762–2769. https://doi.org/10.1039/c6mb00289g
Thompson-Crispi K, Atalla H, Miglior F, Mallard BA. 2014. Bovine mastitis: frontiers in immunogenetics. Front Immunol. 5(OCT):493. https://doi.org/10.3389/fimmu.2014.00493
Wang M et al. 2024. Multi-omics integration identifies regulatory factors underlying bovine subclinical mastitis. J Anim Sci Biotechnol. 15(1):46. https://doi.org/10.1186/s40104-024-00996-8
Xia Y et al. 2018. Betaine inhibits interleukin-1β production and release: potential mechanisms. Front Immunol. 9(Nov):2670. https://doi.org/10.3389/fimmu.2018.02670
Zhang G et al. 2022. Identification of serum-predictive biomarkers for subclinical mastitis in dairy cows and new insights into the pathobiology of the disease. J Agric Food Chem. 70(5):1724–1746. https://doi.org/10.1021/acs.jafc.1c07281
Zhao G et al. 2018. Betaine in inflammation: mechanistic aspects and applications. Front Immunol. 9(May):1070. https://doi.org/10.3389/fimmu.2018.01070
Zhao N et al. 2022. Betaine protects bovine mammary epithelial cells against LPS-induced inflammatory response and oxidative damage via modulating NF-KB and Nrf2 signalling pathway. Ital J Anim Sci. 21(1):859–869. https://doi.org/10.1080/1828051X.2022.2070035
Zhu C et al. 2023. Metabolomic analysis of multiple biological specimens (feces, serum, and urine) by 1H-NMR spectroscopy from dairy cows with clinical mastitis. Animals. 13(4):741. https://doi.org/10.3390/ani13040741

Zhu C, Petracci M, Li C, Fiore E, Laghi L. 2020. An untargeted metabolomics investigation of Jiulong Yak (*Bos grunniens*) meat by 1H-NMR. Foods. 9(4):481. https://doi.org/10.3390/foods9040481

Zwierzchowski G, Haxhiaj K, Wójcik R, Wishart DS, Ametaj BN. 2024. Identifying predictive biomarkers of subclinical mastitis in dairy cows through urinary metabotyping. Metabolites. 14(4):205. https://doi.org/10.3390/metabo 14040205