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^{1}H NMR metabonomics and immune cell signature of milk may reveal insights into subclinical mastitis and quarter interdependence

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ABSTRACT

Milk metabolome depends on a plethora of factors and on the presence of different cell types and could help understanding the biology of the mammary gland and, possibly, identifying biomarkers for mastitis, tissue repairing and milk quality. To fulfill these expectations, metabolome changes need an accurate characterization under several well-characterized physiological and pathophysiological conditions. The aim of the present work is to study mammary quarters of dairy cows affected by subclinical mastitis (SCM) and acute inflammation compared to healthy animals. The milk metabolome was investigated by ^{1}H NMR spectroscopy and by the assessment of somatic cell populations by flow cytometry using a panel of leukocyte markers (CD11b, CD44, CD14, CD4, CD8, CD21). The study was integrated by microbiological evaluations and oxidized proteins (AOPP) determination and results were analyzed by multivariate model. Mammary quarters with the highest CD11b positive cells, suggestive of acute inflammation, were present in SCM-affected cows only, and were characterized by significantly higher AOPP, where the microbiological analysis revealed the presence of minor pathogens. A good PCA separation between healthy and SCM-infected animals was observed (overall error rate: 0.177 ± 0.056) confirming that SCC are associated with modifications of milk metabolome. The classification accuracy was lower (overall error rate: 0.343 ± 0.029) when the mammary quarters were classified on the fraction of CD11b positive cells of quarters from healthy and SCM-affected cows. Interestingly, low-CD11b-SCM samples tended to be misclassified (error rate: 0.460), suggesting the influence of infected neighboring quarters. The results of this work underlay the importance of studying the functional interdependence of mammary quarters in animals affected by SCM.

Simple summary: This study explored how subclinical mastitis—a mild, often hidden udder infection in dairy cows—affects the metabolites in milk. Nuclear magnetic resonance spectroscopy was used for comparing milk from healthy cows to that produced by cows with subclinical mastitis caused by bacterial infections. We found that the metabolic profile (metabolome) of analyzed milk changed noticeably in infected cows. These changes were linked to both immune cell activity and possible damage to udder tissue. Surprisingly, even parts of the udder that seemed healthy in infected cows sometimes showed altered milk composition. This suggests that infections in one part of the udder can influence nearby quarters or cause broader changes in the cow's immune and metabolic systems. The findings support the suggestion that milk metabolites could be used to detect early stages of udder infections. The study also highlights how different quarters of the udder are connected, and how even the "healthy" ones can be affected when a cow suffers from mastitis.

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1. Introduction

The individuation of effective biomarkers of physiological and pathophysiological processes is extremely relevant in dairy sciences for productive, health and welfare purposes and, in the context of the dairy industry and one health, for milk quality assessment (De Almeida et al., 2019; Zachut et al., 2020a;Hu et al., 2021). As an example, mastitis significantly contributes to the direct costs associated with cow diseases and has an impact on public health due to its link with the use of antimicrobials (Akers and Nickerson, 2011; Trevisi et al., 2014; Hughes and Watson, 2018)

Over the years, an increasing amount of information has been collected about pathophysiological processes, diagnosis and control of mammary infections. However, early diagnosis of subclinical mastitis is still complex, and there is the need to increase our understanding on the response of the mammary microenvironment (mammary epithelial cells, fibroblasts, endothelial cells, stroma and immune cells) to invading pathogens during disease development, and post-infection during tissue repairing (Hughes and Watson, 2018).

The analysis of metabolite composition of body fluids can provide useful insights into the health or disease state of an animal and, in dairy cows, metabolomics is increasingly used to investigate disease etiology, develop screening of disease biomarkers, and predict and monitor the treatment of complex diseases (Hailemariam et al., 2014a; Hailemariam et al., 2014b; Imhasly et al., 2014; Li et al., 2014; Zhang et al., 2013; Sun et al., 2014;). In particular, diagnostic possibilities offered by ^{1}H NMR metabolomics and by the characterization of changes of the concentration of small metabolites ($<$1500 Da) in response to modifications of the animals' physiological states attracts scientists into the field (Li et al., 2017a; 2017b; Li et al., 2024).

In dairy animals, milk can be considered the preferred biological matrix for biomarker research (De Almeida et al., 2019; Zachut et al., 2020b; Hu et al., 2021;), as automated non-invasive sampling techniques and monitoring technologies are already available and others will be developed in the near future (Caja et al., 2016).

The secretion of milk components is a complex mechanism involving different secretory pathways (Shennan and Peaker, 2000; Hu et al., 2021). Therefore, when examining variation in milk metabolome, it is important to bear in mind that milk metabolites have different origins (Hu et al., 2021) and the metabolome depends on several factors, such as the animal's physiological phase (e.g., dry period length and lactation stage), energy balance (Lu et al., 2013; Xu et al., 2020a, 2020b) and the presence of mammary infections (Xi et al., 2017; Johnzon et al., 2018). Moreover, infections and other stressful or pro-inflammatory conditions, such as heat stress, can alter the junctional complex function, which regulates the paracellular transport of ions and small molecules across the blood-milk barrier of the mammary gland during lactation (Tao et al., 2018) leading to increased permeability and leakage of metabolites through the barrier itself (Bannerman, 2009; Kobayashi et al., 2013; Yue et al., 2020). In addition, milk metabolites can be secreted or released by commensal and pathogenic microorganisms (Hettinga et al., 2008; Tong et al., 2019; Wang et al., 2022), besides by cells present in milk and mammary tissue (Shennan and Peaker, 2000; Sundekilde et al., 2013). Cells of the mammary epithelium are responsible for the secretion of the largest portion of milk metabolites (Shennan and Peaker, 2000), and changes of the concentration of minor milk constituents may reflect modifications of mammary gland metabolism (Silanikove et al., 2014). Interestingly, as mammary epithelial cells are also effectors of mammary immunity (Mazzilli and Zecconi, 2010), they can modify their metabolic pathways in response to infections (Huang et al., 2019). In an infected mammary gland, epithelial cells dying by necrosis or by secondary necrosis following apoptosis release their cytoplasm into milk (Zhao and Lacasse, 2008), altering the metabolome. Among the mammary gland cells, leukocytes can have a great impact on milk metabolome.

Recently, it has been suggested that the increase in Somatic Cell Count (SCC) can be associated with changes in milk metabolome, which could lead to the identification of metabolites as novel indicators of udder health status and milk quality (Bobbo et al., 2022). Indeed, increased SCC in milk was associated with the presence of several metabolites, even though their exact origin was not determined (Mazzilli and Zecconi, 2010; Bobbo et al., 2022). Somatic cells primarily consist of leukocytes (lymphocytes, monocytes and neutrophils), whose number and proportion are influenced by parity, lactation phase, presence of mammary infection, etiological agent, and the phase of infection (Schwarz et al., 2011; Halasa and Kirkeby, 2020; Zecconi et al., 2020), as well as by cow genetics (Heringstad et al., 2006). Moreover, different cell types may affect milk metabolome in different ways. Neutrophils and monocytes perform phagocytosis at the site of infection and, by releasing enzymes and microbiocidal compounds, such as re-active oxygen species and hypochlorite (Bordignon et al., 2014; Lauvau et al., 2014), can also oxidize host biomolecules. Monocytes and neutrophils die upon spontaneous apoptosis and necrosis, and neutrophils can undergo NETosis (the release of DNA forming extracellular traps capable of killing the invading pathogens (Sladek and Rysanek, 2010; Colitti et al., 2019), releasing their cytoplasm into milk.

In short, milk metabolome depends on a plethora of different factors and on the action of different cell types reflecting the pathophysiological state of the cow, and the study of the whole metabolome could help understanding the biology of the mammary gland and, perhaps, identifying biomarkers for mastitis, tissue repairing and/or milk quality (Li et al., 2017a, 2017b; Hu et al., 2021). However, a biomarker, as well as the whole metabolome, serves to this role only if its relationships with the normal and/or pathophysiologic biological process are fully understood. To achieve readiness for application and to be able to represent the real picture of a biological process, biomarkers need accurate characterization and constant re-evaluation (Puntmann, 2009). For these reasons, milk metabolome modifications should be studied under several well-characterized physiological and pathophysiological circumstances (Hu et al., 2021).

As most studies on milk metabolomics focused on the whole milk, which may lead to the loss of some important biological information about local phenomena occurring within the mammary quarters, we considered it as worthwhile to focus on the metabolome within the individual mammary quarter. In this study, we hypothesized that milk metabolome is affected by the inflammatory conditions related to subclinical mastitis (SCM) and that activated neutrophils exert a major effect in the inflamed mammary quarters. To test this hypothesis, the metabolome of milk samples taken from individual mammary quarters was investigated by ^{1}H NMR spectroscopy. Milk samples were characterized by the analysis of the fractions of somatic cell populations, integrated by microbiological evaluations.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich: anhydrous K_2HPO_4 and anhydrous KH_2PO_4 ($\geq$ 99 %), sodium azide (NaN_3), 99.98 % D_2O, 2,2,3,3-d4–3-(trimethylsilyl)-propionic acid sodium salt (TSP-d4, 98 % D atom) and 99.8 % methanol-d4 (used for NMR temperature calibration).

2.2. Herd management, animal enrolment and milk sampling

The trial was conducted in a dairy farm located in the Veneto region, north eastern Italy. The herd consisted of 135 Italian Holstein Friesian and Italian Simmental lactating dairy cows. Animals were housed in a free stall barn with cubicles, and were fed a total mixed ration consisting of corn silage, corn flour, alfalfa, hay and ryegrass. Moreover, they received a concentrate supplement at each voluntary milking (3.5 kg/head/day). Water was available ad libitum.

Cows were milked with an automatic milking system (DeLaval VMS™) equipped with an online cell counter, and daily monitored for SCC. The farm veterinarian daily assessed the status of the cow udders, which was defined on both SCC and of presentation of clinical signs. Animals presenting obvious clinical signs of acute mammary inflammation (swelling, redness, and udder pain, and presence of clots in milk) were discarded from this study. Cows with signs of other diseases and/or with previous episodes of SCM in the ongoing lactation were also excluded from the study.

Dairy herd improvement milk analyses were available for all cows every 40 days and included protein, fat, lactose and casein content, and were carried out by a Milkoscan 7 RM (Foss A/S, Hillerød, Denmark) at the milk lab of the Veneto region Breeder Association (ARAV, Vicenza, Italy). Based on the dairy herd improvement analyses, 30 animals (22 Holstein and 8 Simmental cows) with diverging milk SCC were enrolled in the study. The selected cows were between 68 and 143 days in milk (DIM), between 1 and 6 parity, and produced on average 39.23 ± 8.12 kg/day of milk, with a protein and fat content of 3.39 ± 0.24 and 3.82 ± 0.64 %, respectively. Somatic cell count varied between 8000 and 1928,000 cell mL-1.

2.3. Milk sampling

Milk samples for the study were collected between February and July 2017. For each selected cow, SCC was measured by the milking robot on a milk sample from all four quarters (composite milk). In addition, individual quarter foremilk samples were collected in the morning by the farm veterinarian, as cows accessed the milking robot. Before quarter sample collections, teats were cleaned with 70 % ethanol and the first two spurts of milk were discarded. From each udder quarter, 10 mL aliquots were collected in sterile 14 mL Falcon® tubes, frozen and sent to the microbiologic laboratory of the Department of Veterinary Medicine, University of Milan, Italy, in dry ice for bacteriological analyses. Further 100 mL aliquots from each quarter were collected into two sterile 50 mL Falcon® tubes and were used for flow cytometry analysis, and to obtain whey samples for Advanced Oxidized Protein Products (AOPPs), thiols and ^{1}H NMR analyses. Samples were stored in ice during transportation to the laboratory, and further processing occurred within 6 h. Once at the laboratory, three 1 mL aliquots of milk samples were skimmed by centrifugation at 4000 g for 15 min at 4°C, and whey was obtained by ultracentrifugation at 100,000 g for 30 min at 4°C by a Beckman Coulter® Optima L 90 K ultracentrifuge. Whey aliquots were frozen at −80°C and used for AOPP, thiols and ^{1}H NMR analyses.

2.4. Microbiological analysis

Bacteriological analyses were performed by streaking 0.01 mL of quarter milk samples (QMS) on blood agar plate with 5 % (v/v) bovine blood according to Hogan et al. (1999). The isolates were identified by a Vitek-2™ (BioMèrieux, Lyon, F), a fully automated system performing bacterial identification and antibiotic susceptibility testing (Barry et al., 2003; Meyer et al., 2008). Based on the presence of potentially pathogenic microorganisms, the udder quarters were defined as either negative (NEG) or infected (INF). A quarter was classified as INF if the presence of at least one potentially pathogenic microorganism was detected.

2.5. Flow cytometry

Milk samples were diluted 1:1 with phosphate-buffered saline and centrifuged 15 min at 1500 rpm at 4°C. The creamy layer and supernatant were removed and cells were washed twice with PBS. The cell pellets were re-suspended in 0.5–1.0 mL of RPMI medium (Thermo Fisher Scientific) and calf serum and sodium azide were added based on cellularity, in order to obtain a final cell concentration ranging from 5 to 10 × 103 cell μL^{-1}. Then, cell suspensions (50 μL) were labelled with the following combination of antibodies: CD11bfitc and CD14PE and CD44APC, CD4PE and CD8APC, CD21PE and CD44APC (Table 1). As a control, proper isotype control antibodies were used. After a 30 min incubation at 4°C, samples were analyzed by CyFlow® Space and FloMax® operating software (Sysmex-Partec, Gorlitz, Germany).

2.6. Advanced Oxidized Protein Products (AOPPs) and thiols in whey

The concentration of AOPPs in whey samples was measured by a spectrophotometric method (Bordignon et al., 2014). Briefly, 0.2 mL whey diluted 1:1 in 20 mM phosphate buffer, pH 7.4, were placed in 96-well microplates (Perkin-Elmer Life and Analytical Sciences, Shelton, CT, USA) and mixed with 20 μL acetic acid. The calibration curve was prepared using 0.2 mL chloramine-T (N-chloro-4-methylbenzenesulfonamide sodium salt, Fluka, St. Louis, MO, USA) 0–200 μM dissolved in distilled water as the reference, 10 μL potassium iodide (1.16 M) and 20 μL acetic acid. The absorbance of the reaction mixtures was immediately read at 340 nm in a microplate reader (Victor X4 2030 Multilabel Reader, Perkin Elmer) against blank (0.2 mL of 20 mM phosphate buffer, 10 μL of 1.16 M potassium iodide and 20 μL of acetic acid). The AOPP concentration was expressed as chloramine-T equivalents. The concentration of thiols in whey was measured by a spectrophotometric method based on the reaction of thiol/disulphide groups with the Ellman's reagent, and using cysteine (0.25–1.50 mM, Sigma–Aldrich, Milan, I) as a standard. Absorbance was read at 412 nm. Protein concentration in whey samples was measured by a commercial kit (BCA Protein Assay Kit, Pierce Biotechnology, Rockford, IL, USA) following the manufacturer's instructions and using bovine serum albumin (BSA, Sigma–Aldrich, Milan, I) as a standard.

2.7. ^{1}H NMR analysis

Whey samples were thaw from −80 °C immediately before NMR analysis, quickly centrifuged at 12,000 × g for 5 min at 4 °C and, in order to minimize pH variability, rapidly diluted (80:20 v/v) in potassium phosphate buffer prepared at a final concentration of 1.5 M in H_2O:D_2O (1:1), pH 7.4. The buffer solution contained TSP-d4, 15 mM final concentration, as chemical shift reference for NMR spectra, and sodium azide (NaN_3), 5 mM final concentration, as antibacterial agent. After dilution, the final pH of the whey samples was 7.30 ± 0.02 (mean ± sd). Buffered samples (600 μL) were transferred into 5 mm NMR tubes and immediately inserted into the spectrometer for the spectrum acquisition. ^{1}H NMR spectra were acquired using a Bruker Advance III spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 300.13 MHz proton Larmor frequency. A ^{1}H – 13 C probe (5 mm outer diameter), equipped with Z gradient facility, was used. Two different pulse sequences were used for the acquisition of the one-dimensional (1D) NMR spectra: 1D Noesy sequence and 1D Carr–Purcell–Meiboom–Gill spin-echo sequence. The 1D Noesy sequence was used to record each ^{1}H NMR spectrum without any digital filter, while the 1D Carr–Purcell–Meiboom–Gill sequence used of a digital filter to attenuate the broad signal from macromolecules (e. g. proteins) in the sample, thus permitting a clearer evaluation of low molecular weight species (metabolites). For both the sequences, the wide and intense peak of water was previously suppressed by a pre-saturating zgpr pulse sequence.

Table 1
Antibodies used for the flow cytometry analyses (type, source and working dilution).

Antibody	Clone	Antibody type	Manufacturer	Working dilution
CD11b-FITC	CC126	Mouse IgG2b	GeneTex®	1:100
CD14-PE	TÜK4	Mouse IgG2b	Abcam®	1:25
CD44-APC	IM7	Rat IgG2b	Invitrogen®	1:25
CD4-PE	CC8	Mouse IgG2a	GeneTex	1:50
CD8-APC	CC63	Mouse IgG2a	AbD Serotec®	1:50
CD21-PE	LT21	Mouse IgG1	GeneTex®	1:50

Spectra acquisition and processing were performed by using TopSpin 3.6.4 software (Bruker Biospin). The acquisition parameters were set to 64 or 2048 scans, 64 K data points, 20 ppm (6003 Hz) as spectral width, 4 dummy scan and a relaxation delay of 4 s. All 1D spectra were then processed using a line broadening of 0.3 Hz, with the chemical shifts internally calibrated to the TSP-d4 peak at 0.0 ppm. All NMR experiments were performed at the constant temperature of 310 K, with approximately 0.1 K temperature stabilization. To make sure that all the spectra were acquired at the same constant temperature, after inserting the samples into the probe, 5 min were allowed to pass before starting the spectra acquisition. The assignments of various peaks were based on measured chemical shifts and split patterns of proton signals cross-checked with metabolite NMR databases (BBIO-REFCODE 2 data-base, Bruker Biospin), Bovine Metabolome Database BMDB (www.bovinedb.ca) and literature published data (Li et al., 2017b; Sundekilde et al., 2013a; Sundekilde et al., 2013b).

2.8. Data processing

2.8.1. Cow classification

The cows enrolled in the trial (N = 30) were allocated to two groups based on SCC in the composite milk: healthy (SCC≤100,000 cell mL-1) or affected by subclinical mastitis (SCM; SCC>200,000 cell mL^{-1}). Cows with intermediate SCC values (between 100,000 and 200,000 cells/mL) were explicitly excluded to ensure a clear contrast between health status categories. As an alternative diagnostic criterion, the same 30 cows were diagnosed as either healthy, if all the mammary quarters were NEG, or with intramammary infection (IMI), if at least one mammary quarter was infected (INF). The agreement between the two diagnostic criteria was assessed by the Cohen's kappa coefficient (IBM SPSS Statistics 29).

2.8.2. Udder quarter classification

As the differential cell count in milk somatic cell could provide more information about the dynamics of udder health (Halasa et al., 2020), particularly with respect of the percentage of neutrophils, the udder quarters (N = 117; three animals had a blind quarter) were classified based on the proportion of neutrophils identified by the expression of CD11b integrin, as marker of udder inflammation (De Matteis et al. 2020). CD11b protein plays a crucial role in immune cell adhesion, migration, and phagocytosis, making CD11b positive cells important players in the inflammatory response (Kelm et al., 2020). The CD11b integrin expression in mammary quarters was analyzed by the TwoStep Cluster Analysis procedure (IBM SPSS Statistics 29.0) with the Euclidean distance measure to compute the similarity among clusters, and mammary quarters were allocated into two clusters according to the proportion of cells expressing the CD11b integrin (low vs high). Finally, the obtained CD11b-based clusters were allocated within animals diagnosed as healthy and SCM, resulting in the classification of mammary quarter into three groups: low CD11b in a healthy cow (Low-CD11b-Healthy; N = 50), low CD11b in a SCM cow (Low-CD11b-SCM; N = 41), and high CD11b in a SCM cow (High-CD11b-SCM; N = 26). No quarters classified as high-CD11b were detected in healthy cows.

2.8.3. Analysis of oxidative stress biomarkers and leukocyte populations

The biomarkers of oxidative stress (AOPP, and thiols) and the fractions of cell populations (CD14, CD44, CD4, CD8, CD21,) were tested by analysis of variance using the Generalised Linear Mixed Model option of SPSS (IBM SPSS Statistics 29). The mammary quarter group (Low-CD11b-Healthy, Low-CD11b-SCM, and High-CD11b-SCM), parity (primiparous and multiparous), breed (Holstein and Simmental) and the covariate DIM were the fixed effects, and the cow was the random effect. When an effect was statistically significant ($P < 0.05$), differences among levels were tested by Least Significant Difference pairwise comparisons and shown as Estimated Marginal Means (IBM SPSS Statistics 29).

2.8.4. ^{1}H NMR spectra editing and PCA

To ensure that the spectra produced comparable data in the bucket table's calculation process, the original data from each spectrum have been scaled to their total intensity by Pareto scaling. In order to reduce the data dimensionality and correct for peak shifting (Jellema, 2009), the ^{1}H NMR spectra acquired from milk samples taken from the individual mammary quarters were divided into evenly spaced windows of 0.05 ppm each (bins). The intensities inside each bin were summed, so that the area under each spectral region was used instead of individual intensities. Therefore, a new smaller set of variables (N = 1000, each one being the result of the sum of intensities within each bin) was created. The width of the bins was set to cover the chemical shift variability around the peaks, reducing their misalignment. After elimination of bins which signal intensity was close to 0, had very low variation, or were located within noise regions, the total number of ^{1}H NMR spectra variables was 588. Outlier inspection was performed by PCA, after centering and scaling the variables to a mean equal to 0 and SD equal to 1. The standardized Mahalanobis distance (GH) was then used to determine the distance of each sample from the centroid and, based on this criterion, six samples with GH> 3 were removed as outliers. Hence, 111 samples from 29 cows were used for further analyses. The six identified outliers did not share any specific biological commonality—such as breed, DIM, or SCC.

The samples remaining after outlier elimination were subjected to an additional preliminary PCA to highlight potential differences in the metabolic profile of milk samples across SCC groups (healthy vs SCM), and udder quarter groups (Low-CD11b-Healthy, Low-CD11b-SCM, and High-CD11b-SCM) following an unsupervised approach. The scores of the first 3 PC obtained were used to visually assess the potential presence of clusters between samples.

2.8.5. Multivariate statistical analysis of the ^{1}H NMR spectra for SCM classification

Each of the 588 ^{1}H NMR variables acquired from milk samples taken from the individual mammary quarters was adjusted for the fixed effect of the breed (2 levels: Holstein and Simmental), parity (2 levels: primiparous and multiparous), and lactation stage (3 levels: ≤110 DIM, 110 < DIM ≤ 130, >130 DIM) using a linear model. The residuals of the models were then used in the subsequent analysis. As measures of SCC were obtained from the composite milk and we did not know the value of SCC for each quarter, using spectra from single quarters to classify cows based on the SCC of the composite milk would generate inaccuracies. Consequently, the adjusted ^{1}H NMR spectra were averaged across cows to obtain one spectrum for each animal, merged to the SCC values, and used to classify the cows in healthy and affected by SCM.

In preliminary discriminant analyses, three multivariate prediction algorithms were tested for the prediction of the SCM class (healthy vs SCM): partial least square (PLS-DA), sparse partial least square (sPLS-DA), and orthogonal projections to latent structures (oPLS-DA). Compared to the more commonly used PLS, sPLS is a multivariate technique used in prediction (or classification) particularly suited for small datasets, presence of many irrelevant predictors, high collinearity among candidate predictors and for the sparsity constraint that allows for variable selection (Chun and Keleş, 2010). oPLS is widely used in -omic sciences and was developed as a way to deal with the large amount of variation in predictor matrices for multivariate prediction (or classification), not correlated to the responses. Orthogonal PLS enables to separately model the variation correlated (predictive) to the factor of interest and the uncorrelated (orthogonal) variation (Trygg and Wold, 2002).

The adjusted ^{1}H NMR variables were standardized to mean = 0 and SD = 1 before the analysis. The number of variables tested in sPLS-DA ranged from 20 to 580, with a 20 variable increase at each iteration. The optimal number of components was identified based on the decrease in the prediction error rate provided by each additional component. PLS-DA, sPLS-DA, and oPLS-DA analyses were performed in the R software

(R Core Team, 2023), using the packages "PLS", "MixOmics", and "ropls", respectively, and in-house scripts. The SCM class was modelled as the sole response variable and the adjusted ^{1}H NMR spectra variables as predictors. The prediction accuracy (measured by the R^2) of each method was tested in a 4-fold random cross-validation. In preliminary analyses, the sPLS-DA algorithm provided the highest accuracy and was then selected as the method of choice. The optimal parameters (number of components and number of variables) in the model were identified using a 4-fold random cross-validation repeated for 50 times, based on the maximization of the Mahalanobis distance between groups and minimization of the misclassification error rate.

After model tuning, the optimal number of components selected was 1. The error rate across number of components and number of selected variables for the selected component are reported in the Supplementary material (Figure S1 a, b).

The final sPLS-DA models used the tuned parameters (1 component and 160 selected variables). The overall error rate (measured as the proportion of correctly identified samples to the total number of samples) and the balanced error rate (measured as the average proportion of wrongly classified samples in each class, weighted by the number of samples in each class) of the final model were tested in a 4-fold random cross-validation and used as measures of model accuracy.

The variable importance in projection (VIP) scores were estimated to identify the spectral variables that mostly contributed to the prediction. They reflect both the loading weights for each component and the variability of the response explained by this component (Mehmood et al., 2012; Pinto et al., 2012). Commonly, a variable with a VIP score greater than 1 is considered important in a given model (Chong and Jun, 2005). The variables with the 12 highest VIP scores, representing the spectral variables contributing the most to the variance between groups, were identified and discussed.

2.8.6. *Multivariate statistical analysis of the ^{1}H NMR spectra for mammary quarter groups*

The ^{1}H NMR variables were also used to classify mammary quarters into the three groups Low-CD11b-Healthy, Low-CD11b-SCM, and High-CD11b-SCM. Analogously to the classification of SCM, each of the ^{1}H NMR variables was adjusted for the fixed effect of the breed, parity, and lactation stage. Additionally, as repeated observations were available for each cow, the random effect of the cow (29 levels) was also tested, but it was not significant for the large majority of the variables and it was excluded from the final model. The adjusted ^{1}H NMR spectra were used to classify the mammary quarters in the three groups. Analogously to the classification of SCM, sPLS-DA outperformed PLS-DA and oPLS-DA and was used as the method of choice. The quarter class was modelled as the response variable with the adjusted ^{1}H NMR spectra variables as predictors. After model tuning, the optimal number of components selected was 5. The error rate across number of components and number of selected variables for the selected components are reported in the Supplementary material (Figure S2 a, b).

3. Results

3.1. *Diagnosis of subclinical mastitis and intramammary infection*

Based on SCC in the composite milk, 13 cows were classified as healthy and 17 as SCM. Pathogenic microorganisms were isolated from 19 mammary quarters (Table 2), and none of the cows classified as healthy based on SCC displayed INF quarters. In 7 out of 17 SCM cows, no pathogenic microorganisms were isolated from any mammary quarter. The diagnosis of SCM based on SCC in composite milk showed a moderate level of agreement (Cohen's kappa = 0.553 ± 0.132, T = 3.387, $P < 0.001$) with that of IMI, based on the presence of a pathogen in at least one mammary quarter.

Table 2

Microorganisms isolated from the mammary quarters of dairy cows (N = 30) diagnosed as Healthy (SCC≤100,000 cell/mL) or affected by Sub-Clinical Mastitis (SCM; SCC>200,000 cell/mL) based on SCC in the composite milk from all quarters. Three cows had a blind quarter.

Microorganism	Mammary quarters (N)		
	Healthy cows	SCM cows	Total
Negative	50	48	98
Escherichia coli	0	1	1
Enterococcus faecalis	0	2	2
Kokuria rosea	0	1	1
Lactococcus garviae	0	3	3
Staphylococcus chromogens	0	8	8
Staphylococcus chromogens + *Staphylococcus xylosus*	0	1	1
Staphylococcus sciuri	0	1	1
Staphylococcus simulans	0	1	1
Staphylococcus warnerii	0	1	1
Total	50	67	117

3.2. *Whey oxidative stress biomarkers and milk cell sub-populations*

In healthy cows, all mammary quarters displayed a low fraction of CD11b positive cells, while SCM cows bore at least one mammary quarter with high proportion of CD11b positive cells. A significant effect of the mammary quarter group was observed on AOPP, CD21 ($P < 0.001$), CD14 ($P < 0.01$), CD44, and CD8 ($P < 0.05$) positive cells (Table 3).

3.3. *Milk metabolome by ^{1}H NMR and spectra adjustment*

The evaluation of the ^{1}H NMR spectra for the assignment of the peaks appearing in the milk samples allowed the recognition of 39 metabolites (Fig. 1). In alphabetical order, the identified metabolites are the following: acetate, acetyl-carnitine, alanine, arginine, β-hydroxy-butyric acid (BHB), butyrate, carnitine, choline, citrate, creatine, creatinine, dimethyl sulfone, ethanol, formate, fucose, fumarate, galactose, 1-phospho-galactose, glucose, 1-phospho-glucose, glutamine, glutamate, glycero-phosphoryl-choline, hippurate, isoleucine, lactate, lactose, leucine, malate, N-acetyl-carbohydrates, N-acetyl-glucosamine, orotate, oxoglutarate, phosphocholine, phosphocreatine, proline, succinate, UDP-N-acetyl-glucosamine and valine.

The effects of cow breed, parity, and lactation stage jointly explained on average 8.2 ± 7 % of the variability of the ^{1}H NMR variables, with R^2 of the linear models ranging from 0.3 % to 53 %. In 25 % of the ^{1}H NMR variables, values of R^2 were higher than 10 %.

3.3.1. *Cow classification from ^{1}H NMR spectra*

When PCA was applied to the adjusted milk ^{1}H NMR spectra, the first 10 principal components accounted for 84.8 % of the spectral variation. In particular, the first three principal components accounted for 27, 16.9, and 10 % of the total spectral variance, respectively (data not reported). PCA scores did not show a visible clustering, as a consequence of substantial overlapping between their scores of healthy and SCM cows (data not reported).

Scores of the sPLS-DA for the classification of cows in healthy and SCM ensembles, obtained after model tuning, are displayed in Fig. 2. The score plot, based on the sole first sPLS-DA component selected during model tuning, showed a good separation between the two groups, with some level of overlapping. sPLS-DA accuracy in classification, as measured by the overall and balanced error rate, is reported in Table 4. The good separation between groups was confirmed by a low overall and balanced error rate of the model (0.177 ± 0.056 and 0.173 ± 0.055, respectively), indicating a high accuracy of the model in classification. The error rate was higher for the SCM than for the healthy group (0.204 vs 0.143), indicating that a larger proportion of SCM samples tended to

Table 3
Biomarkers of oxidative stress and proportions of the cell populations measured in the milk samples from individual mammary quarters.

	Estimated Marginal Means ± s.e.m.			GLMM Fixed effects (F)			
	Mammary quarter group						
Biomarker	Lo-H (N = 50)	Lo-SCM (N = 41)	Hi-SCM (N = 26)	Mammary quarter group	Parity	Breed	DIM
AOPP (nmol/mL)	112.3 ± 11.2^{a}	117.4 ± 10.4^{a}	149.0 ± 10.9^{b}	7.231***	0.002	11.673***	0.028
Thiols (nmol/mL)	108.4 ± 17.4	117.2 ± 17.4	95.3 ± 19.3	0.531	1.010	0.960	0.005
Cell populations							
CD 14 (%)	10.8 ± 3.3^{ab}	7.2 ± 3.0^{a}	14.8 ± 3.1^{b}	6.648**	1.849	0.000	2.958
CD 44 (%)	60.5 ± 9.1^{ab}	47.2 ± 8.6^{a}	56.9 ± 8.7^{b}	4.548*	3.769	1.491	0.965
CD 4 (%)	3.6 ± 1.0	3.5 ± 0.9	3.1 ± 0.9	0.236	1.151	0.212	2.100
CD 8 (%)	7.3 ± 1.5^{b}	4.0 ± 1.3^{b}	2.6 ± 1.4^{a}	4.557*	4.937*	0.457	0.000
CD 21 (%)	0.92 ± 0.14^{b}	0.92 ± 0.13^{b}	0.46 ± 0.14^{a}	7.706***	0.594	1.318	0.941

Mammary quarters were classified based on the health status of the cow (Healthy vs Sub Clinical Mastitis – SCM) and the proportion of CD11b positive cells, as described in Materials and Methods. Three cows (two healthy and one affected by SCM). had a blind quarter. Data were analysed by the Generalised Linear Mixed Models (GLMM) option of SPSS (IBM SPSS Statistics 29), considering the mammary quarter group, parity, breed and the covariate days in milk (DIM) as fixed effects, and the cow as the random effect.
Biomarkers of oxidative stress (AOPP, thiols)
Cell populations (CD14, CD44, CD4, CD8, CD21)
Mammary quarter groups. Lo-H: Low CD11b-Healthy cow; SCM-Lo: Low CD11b-SCM cow; Hi-SCM: High CD11b-SCM cow.
Different letters (^{a,b}) in the same row indicate significantly different means ($P < 0.05$; least significant difference pairwise comparisons). The asterisks indicate statistically significant effects (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

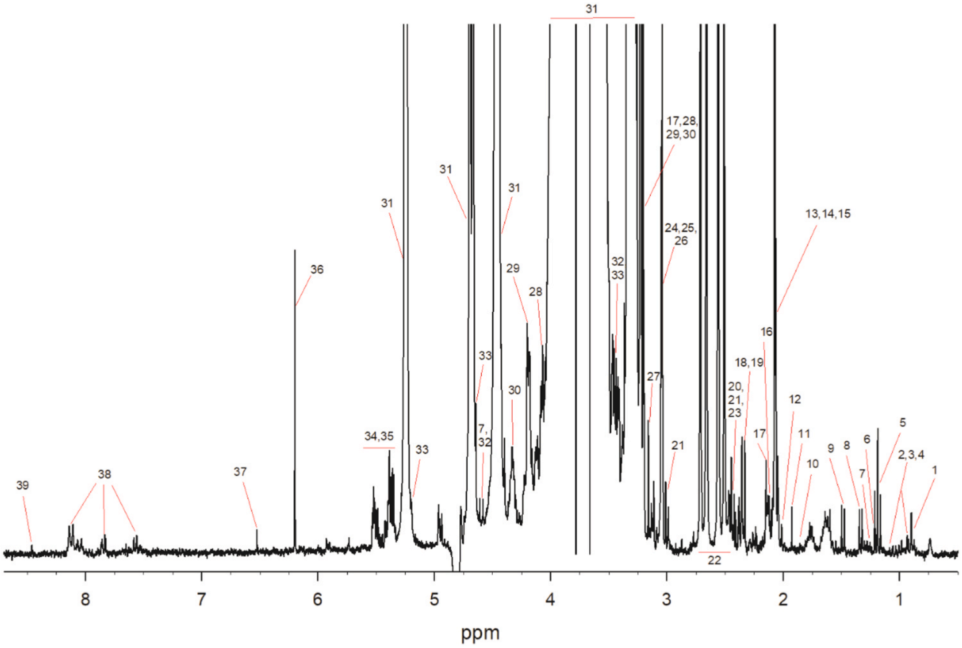

Fig. 1. Typical ^{1}H NMR spectrum obtained from milk samples. 1. Butyrate, 2. Leucine, 3. Isoleucine, 4. Valine, 5. Ethanol, 6. BHBA, 7. Fucose, 8. Lactate, 9. Alanine, 10 Arginine, 11. Acetate, 12 Proline, 13. N-Acetyl Carbohydrates, 14. N-Acetyl Glucosamine. 15 UDP-N-Acetyl Glucosamine, 16. Glutamine, 17. Acetyl Carnitine, 18. Glutamate, 19. Malate, 20. Carnitine, 21. Oxoglutarate, 22. Citrate, 23. Succinate, 24. Creatine, 25. Phosphocreatine, 26 Creatinine, 27. Dimethyl sulfone, 28 Choline, 29. Phosphocholine, 30 Glycero-phpsphocholine, 31. Lactose, 32. Galactose, 33 Glucose, 34. Glucose-1P, 35. Galactose-1-P, 36. Orotate, 37 Fumarate, 38. Hyppurate, 39. Formate.

be misclassified as healthy samples.

The 12 ^{1}H NMR bins that most contributed to the classification of healthy and SCM group are reported in Fig. 3. Metabolites could be identified in most of the bins, but for some bins no metabolites were detected because of unclear identification of hyperfine splitting and lack of specific literature data which could be used for comparison. Hence, metabolites within these bins were listed as “Unknown”. In addition, some spectral regions corresponded to multiple metabolites. Therefore, only the most representative ones were listed. Compounds identified in the NMR spectral bins with the highest VIP score for the prediction of SCC groups were arginine, butyrate, acetyl-carbohydrates and N-acetylglucosamine. Arginine and butyrate were associated to multiple spectral bins with high VIP score, confirming their importance in the classification of cows in healthy or SCM groups.

An increased signal intensity of all the 12 bins (corresponding to a higher concentration of identified metabolites) was associated to a higher probability of a milk sample to be classified as SCM, as indicated by the distribution of the ^{1}H NMR signal across SCC groups (Fig. 4).

Overall error rate (± SD), balanced error rate (± SD), and error rate across groups, in classification of cows in healthy and affected by subclinical mastitis (SCM), and, in classification of mammary quarters in three classes according to the content of CD11b (high vs low) and absence or presence of subclinical mastitis (healthy vs SCM, respectively). Cows were classified as healthy when SCC< 100,000 cells mL-1,

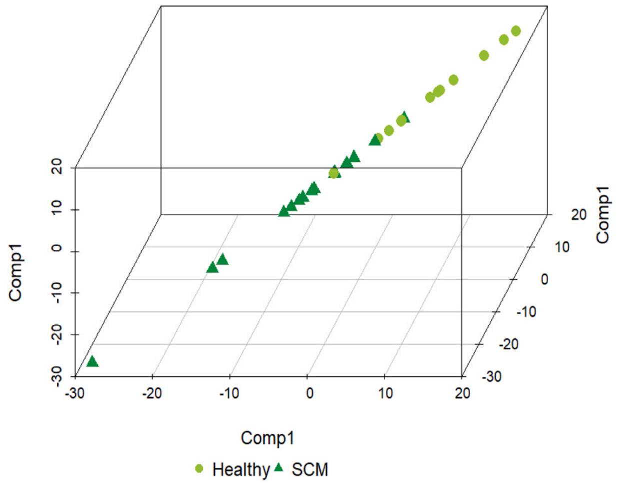

Fig. 2. ^{1}H NMR spectra score plots for the first sPLS-DA component after model tuning. Each point represents a cow's milk sample. Cows were classified in two classes according to the SCC (healthy vs affected by subclinical mastitis, SCM). Cows were classified as healthy when SCC< 100,000 cells mL-1, and were classified as SCM when SCC> 200,000 cells mL-1).

Table 4
Error rates of models.

Error term	Error rate	
	Healthy vs SCM cows	Mammary quarters
Overall error rate	0.177 ± 0.056	0.343 ± 0.029
Balanced error rate	0.173 ± 0.055	0.357 ± 0.030
Error rate in the Healthy group	0.143	–
Error rate in the SCM group	0.204	–
Error rate in the Low-CD11b-Healthy group	–	0.244
Error rate in the Low-CD11b-SCM group	–	0.460
Error rate in the High-CD11b-SCM group	–	0.368

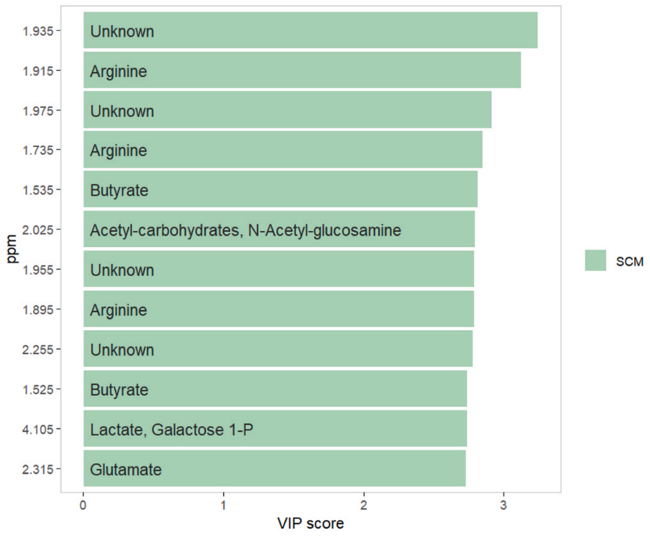

Fig. 3. Metabolites associated with the presence of subclinical mastitis. Metabolites associated with the bins with the 12 highest values of variable importance in projection (VIP) scores in the classification of cows in healthy or affected by subclinical mastitis (SCM). For all bins, high values of intensity were associated with an increased probability of SCM.

and as affected by SCM when SCC> 200,000 cells mL-1).

3.3.2. *Mammary quarter groups classification by ^{1}H NMR spectra*

The first 10 principal components of the adjusted mammary quarter spectra accounted for 76.1 % of the spectral variation, with the first three principal components accounting for 30.1, 19.8, and 6.5 % of the total spectral variance, respectively (data not reported). Quarter classification did not result in a visible PCA clustering (data not reported). Conversely, the scores of the sPLS-DA displayed a good degree of separation among groups, with some overlapping occurring especially between groups Low-CD11b-Healthy and Low-CD11b-SCM (Fig. 5).

The accuracy of sPLS-DA model classification was lower than that obtained for cow classification in healthy and SCM, with an overall error rate of 0.343 ± 0.029 (Table 4). Of the mammary quarter groups, the Low-CD11b-SCM exhibited the highest error rate (0.46), indicating that 46 % of those samples tended to be misclassified as Low-CD11b-Healthy or High-CD11b-SCM samples.

The 12 ^{1}H NMR bins most contributing to the classification of the udder quarter into the three groups (Low-CD11b-Healthy, Low-CD11b-SCM or High-CD11b-SCM), are reported in Fig. 6. Here, the compounds identified in the ^{1}H NMR spectral bins with the highest VIP score for the prediction of SCC groups largely overlapped with those observed for cow classification in healthy and SCM (i.e., arginine, butyrate, acetyl-carbohydrates, and N-acetyl-glucosamine). In addition to these metabolites, also valine and proline played a significant role in mammary quarter classification.

The distribution across mammary quarter groups of ^{1}H NMR signal for each bin with the highest VIP score is reported in Fig. 7. Overall, an increasingly higher signal intensity for all the bins with the highest VIP scores was observed in Low-CD11b-Healthy, Low-CD11b-SCM, and High-CD11b-SCM, with the latter exhibiting the highest intensity values. Signal intensity was particularly high in the High-CD11b-SCM group, with Low-CD11b-Healthy and Low-CD11b-SCM exhibiting similar values. The High-CD11b-SCM group was also characterized by the highest variability of signal intensity.

4. Discussion

4.1. *Sample classification*

In the present study, milk metabolome was investigated in individual mammary quarters by ^{1}H NMR spectroscopy by a metabonomic approach. To limit any misclassification of cows potentially affected by SCM in the healthy group, animals were considered as healthy if SCC in the composite milk from all quarter was lower than 100,000 cells/mL, while cows were classified as SCM-affected if SCC in the bulk milk was higher than 200,000 cells/mL. As samples were collected for experimental purposes, no comparison of SCM prevalence with larger epidemiological studies could be done [e.g.: Zecconi et al., 2019)].

The microbiological analysis allowed the assessment of the potential role of bacterial infections on the pathophysiological state of the animals, and showed the presence of minor pathogens in 16.2 % of the mammary quarters belonging to the SCM-affected cows. The classification based on the presence of IMI showed only a moderate degree of agreement with that based on SCC. Furthermore, a PLS analysis based on the presence of IMI did not show any clustering (data not shown). In any case, the heterogeneity of the isolated microorganisms combined with the low number of IMI positive samples did not allow any comparison with other published data.

4.2. *Role of somatic cells*

The predominant early nature of the inflammatory processes within the individual mammary quarters was assessed by the analysis of the fractions of somatic cell populations. We classified the inflamed mammary quarters as those exhibiting a high fraction of CD11b positive cells,

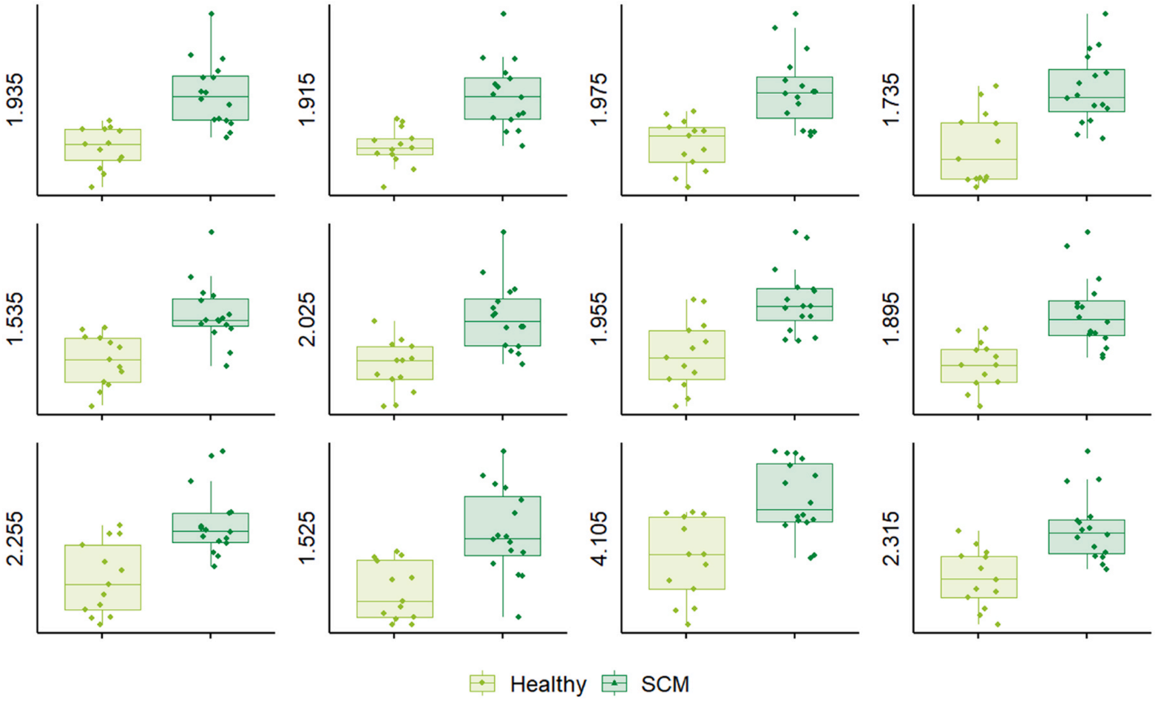

Fig. 4. Distribution of the ^{1}H NMR signals across healthy cows and cows affected by subclinical mastitis. The bins with the 12 highest values of variable importance in projection scores across cow groups (healthy or affected by subclinical mastitis, SCM). Cows were classified as healthy when SCC< 100,000 cells mL-1, and as affected by SCM when SCC> 200,000 cells mL-1).

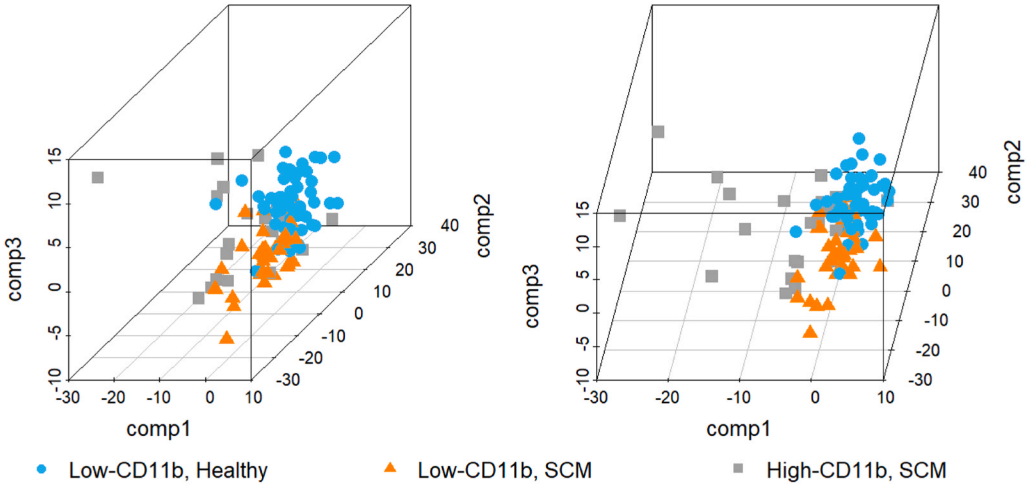

Fig. 5. ^{1}H NMR spectra score plot for the first three components of the sPLS-DA model after tuning. The score plot is displayed from two different angles. Each point represents a quarter foremilk sample. Samples were classified in three classes according to the content of CD11b (high vs low) and absence or presence of subclinical mastitis (healthy vs SCM, respectively). Cows were classified as healthy when SCC< 100,000 cells mL-1, and as affected by SCM when SCC> 200,000 cells mL-1).

suggesting the presence of high proportions of neutrophil and macrophages (Borjesson et al., 2002; De Matteis et al. 2020) and an early condition of inflammation without clinical signs. The CD11b antigen is part of macrophage-1 antigen (Mac-1), a heterodimer consisting of αM (CD11b) and β2 (CD18) proteins, which are expressed in myeloid cells, such as monocytes-macrophages, eosinophils, neutrophils, and basophils, and in lymphoid cells, such as NK cells, and peritoneal B-1 cells (Mitroulis et al., 2015). Mac-1, normally expressed in circulating leukocytes in an inactive form, binds to different ligands, (Otterlei et al., 1993) including ICAM-1 (CD54), ICAM-2 (CD102) and fibrinogen, and it can be rapidly activated by chemokines for mediating the adhesion to endothelial cells. Moreover, Mac-1 binds to iC3b of the complement system promoting phagocytosis of opsonized bacteria. Thus, CD11b antigen can be considered a reliable indicator of subclinical mastitis in milk. In addition, we considered the expression of the CD44 glycoprotein, which plays a role in the mediation of blood macrophages and PMN recruitment in response to inflammatory signals (Gonen et al., 2008). This receptor is expressed in response to LPS (lipopolysaccharide) and MDP (muramyl dipeptide), in particular during initiation of the inflammatory response (Sladek and Rysanek, 2009, 2010). Finally, we considered the presence of the CD14 antigen, a surface receptor of neutrophils and macrophages membranes, which displays recognition potential for Gram-negative and Gram-positive bacteria (Otterlei et al., 1993). It is worth noting that, in response to LPS, CD14 expression in neutrophils and macrophages depends on the stage of mammary gland inflammation in response to a challenge, with a reduced expression in

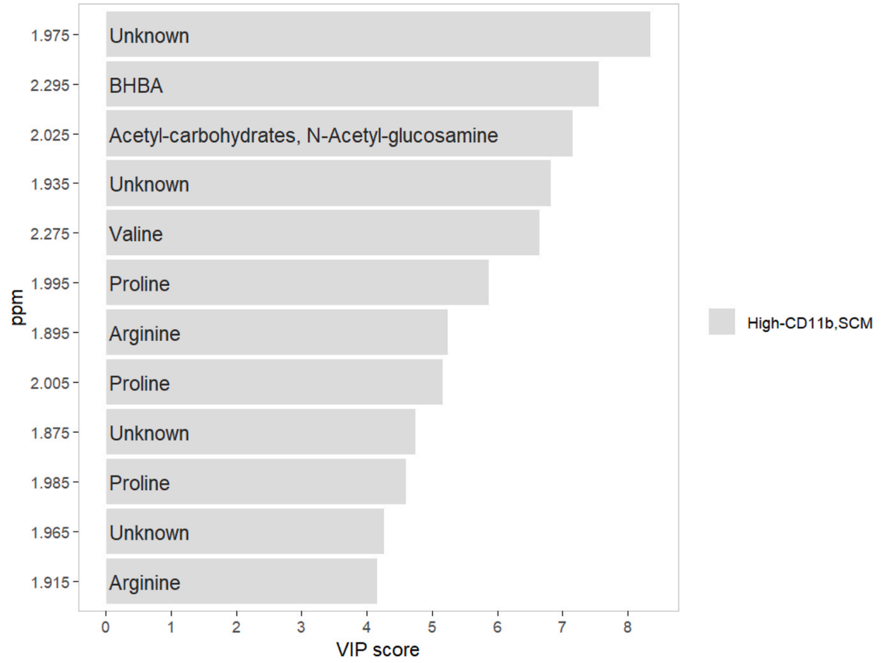

Fig. 6. Metabolites associated with the content of CD11b and the presence of subclinical mastitis. Metabolites associated with the with the bins with the 12 highest values of variable importance in projection (VIP) scores in the classification of udder quarters in according to the content of CD11b (high vs low) and absence or presence of subclinical mastitis (healthy vs SCM, respectively). Cows were classified as healthy when SCC< 100,000 cells mL-1, and as affected by SCM when SCC> 200,000 cells mL-1). For all bins, the highest values of intensity were associated with an increased probability of High-CD11b-SCM.

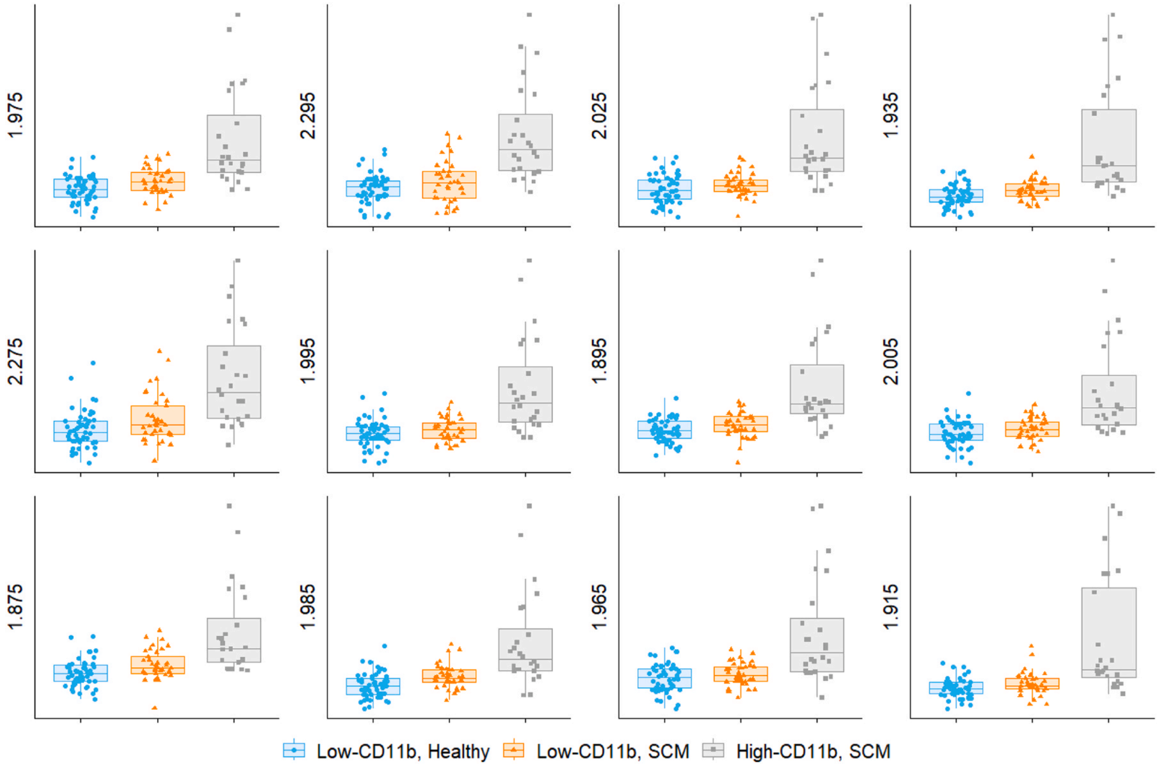

Fig. 7. Distribution of the ^{1}H NMR signal across cow groups and CD11b levels. Distribution of the ^{1}H NMR signal of the bins with the 12 highest values of variable importance in projection scores in the samples with low CD11b in healthy cows (SCC<100,000 cells mL-1), low CD11b, but in cows affected by SCM (SCC>200,000 cells mL-1), and high CD11b in cows affected by SCM.

the early stage (Paape et al., 1996), followed by an increase in the resolution stage (Sládek et al., 2002). Changes of the total and relative count of both CD14 positive neutrophils and macrophages were observed following the infection of mammary glands by *S. aureus* and *S. uberis* (Sladek and Rysanek, 2006). The initial response was characterized by the increase of total counts of CD14 positive neutrophils and

CD14 positive macrophages, while resolution was accompanied by an increase of relative counts of CD14 positive neutrophils and vacuolated macrophages (Sladek and Rysanek, 2006). Considering the high level of CD14 positive cells observed in the High-CD11b-SCM group in this trial, it is possible to hypothesize that a few High-CD11b-SCM samples reflected the initial resolution phase.

A further indication of the early inflammatory stage of High CD11b-SCM quarters relays on their high milk AOPP concentration. AOPPs in plasma are considered a biomarker of activated neutrophils, which release myeloperoxidase (MPO) catalyzing the formation of hypochlorous acid, a potent oxidant (Bordignon et al., 2014). The hypochlorous acid released by neutrophils plays an important role in killing invading pathogens, but its excessive or misplaced generation during inflammation can cause tissue damage, cytolysis and increased permeability of the blood-milk barrier (Zhao and Lacasse, 2008; Akers and Nickerson, 2011; Colitti et al., 2019). Therefore, products of whey protein oxidation result from an accumulation of oxidative insults between consecutive milkings (Celi and Gabai, 2015) and can be considered a biomarker of neutrophil and phagocyte activation in milk, as well as in blood plasma.

CD8 positive T-cells and CD21 positive B cells fractions were lower in quarters with high CD11b SCM-affected cows. A higher fraction of CD21 positive B cells was observed in uninfected quarters from infected udders (Blagitz et al., 2015), corroborating our observation. Conversely, the same authors did not observe any difference in CD8 positive cells between infected and non-infected quarters (Blagitz et al., 2015). In our experiment, pathogens isolated from the tested samples were Gram-positive and Gram-negative bacteria, which usually induce a first innate immune response driven by neutrophils, explaining the relative decrease of CD8 and CD21 lymphocytes in samples with the highest fraction of CD11b cells. Indeed, lymphocytes and macrophages represent the main actors of adaptive immune response and chronic inflammation, respectively. Thus, they are expected to dominate in infections due to pathogens eliciting this kind of immune response, such as *Prototheca* spp. (Pegolo et al., 2022) or in chronic processes (Leitner et al., 2000).

4.3. ^{1}H NMR metabonomics

Regarding results obtained by ^{1}H NMR spectroscopy, although spectra by a 300 MHz NMR spectrometer have a lower resolution compared with those generated by instruments with higher magnetic fields, they contain a wide range of metabolite data, which can subsequently undergo multivariate statistical evaluation and can provide a view of the biological processes at a specific time-point (Percival et al., 2021). Whey ^{1}H NMR spectra were statistically analyzed following two criteria of classification. First, cows were classified in two groups (healthy vs SCM affected animals) based on the SCC in the composite milk. Then, the mammary quarters were classified in three groups, based on the proportion of CD11b positive cells in mammary quarters from healthy and SCM-affected cows.

The sPLS-DA analysis of the whey ^{1}H NMR spectra showed differences between the experimental groups. In particular, the score plot, based on the sole first sPLS-DA component selected during model tuning, showed a good separation between healthy and SCM-infected animals, with some level of overlapping, as suggested by the low overall and balanced error rate of the model (0.177 ± 0.056 and 0.173 ± 0.055, respectively). If the model allocated samples to each of the two groups randomly, this would correspond to an overall error rate equal to 0.5. Hence, the error rate obtained was significantly lower than a random allocation of the samples to the two groups. The error rate was higher for the SCM than for the healthy group (0.204 vs 0.143), indicating that a larger fraction of SCM samples tended to be misclassified as healthy samples. Therefore, this study confirms that increased SCC is associated with changes of milk metabolome (Sundekilde et al., 2013; Luangwilai et al., 2021; Bobbo et al., 2022; Zhu et al., 2024).

When ^{1}H NMR spectra of mammary quarters were classified in three groups based on the fraction of CD11b positive cells from healthy and SCM-affected cows, the accuracy of sPLS-DA model was lower, with an overall error rate of 0.343 ± 0.029. The expected error rate of a model that classifies records randomly into three classes can be calculated on the probability of incorrectly classifying a record. If a record is classified randomly, each class has an equal probability of being chosen. Since there are three classes, the probability of randomly selecting the correct class is 0.33. Consequently, the probability of incorrectly classifying a record (the error rate) is the complement of this probability: 0.67. Hence, despite its higher error rate compared to the SCC classification, this model performed significantly better than a random allocation of samples into the three groups.

Interestingly, the Low-CD11b-SCM mammary quarter group exhibited the highest error rate (0.46), indicating that 46 % of those samples tended to be misclassified as Low-CD11b-Healthy or High-CD11b-SCM samples. However, it is important to note that classification into three biologically meaningful categories is inherently more challenging than a classification into two categories, especially when the intermediate group is expected to exhibit transitional or mixed characteristics between healthy and more clearly inflamed states. The Low-CD11b SCM group, by design, is characterized by subtle immunological and inflammatory features, making it more difficult to clearly distinguish from both the healthy and High-CD11b SCM groups. A higher level of misclassification observed in this group was therefore expected and likely reflects the biological complexity and overlapping features of early or mild inflammation. The patterns observed in misclassification actually support the intermediate nature of the Low-CD11b SCM group. This reinforces the biological relevance of our categorization and highlights the potential of our model to capture meaningful variation in intramammary immune status.

4.4. Identification of metabolites

Despite the low resolution of 300 MHz NMR spectra which may lead to overlapping of signals within the same bin, in this experiment the ^{1}H NMR spectra of whey allowed the identification of 39 metabolites. The first twelve bins with a VIP score higher than 1.5 were considered, and only clearly identifiable metabolites in one or more bins are discussed (Jellema, 2009; Percival et al., 2021). No metabolite was identified within six bins belonging to the first twelve VIP score between 1.875 and 2.255 ppm. Specifically, regarding identified metabolites, an increased arginine content was observed in milk from SCM cows and, in particular, in High-CD11b-SCM quarters, where also proline and valine concentrations were increased with respect to healthy samples. Similar findings were reported by ^{1}H NMR at higher resolution (600 MHz) for subclinical (Zhu et al., 2024) and clinical mastitis (Zhu et al., 2021). Moreover, higher levels of valine were observed in milk samples with high SCC (Bobbo et al., 2022) and in both clinical and subclinical mastitis (Luangwilai et al., 2021).

The increased content of several amino acids in the milk of cows affected by both subclinical and clinical mastitis represents a common result of reported studies. Interestingly, alterations of amino acid concentrations were found also in the plasma of SCM cows (Dervishi et al., 2017; Lisuzzo et al., 2024). Thus, it appears that an alteration of amino acid metabolism occurs during and after the mastitis inflammation process (Dervishi et al., 2017), suggesting that the systemic inflammatory response stimulates protein catabolism. This could result in a general increase of the concentration of serum amino acids, providing substrate for the synthesis of proteins involved in the immune response (Dervishi et al., 2017). Alternatively, an increased amount of damaged proteins derived from the inflammation response may stimulate proteolysis in SCM cows. Moreover, in SCM cows was observed an increased concentration of milk lactate and butyrate, and a high ß-hydroxybutyrate content was measured in High-CD11b-SCM mammary quarters. Even the modification of the content of these metabolites appears a common feature in cows affected by IMI (Luangwilai et al., 2021; Zhu et al., 2021), and in cows with high SCC (Sundekilde et al., 2013; Bobbo et al., 2022). Indeed, high milk lactate was observed during both clinical and subclinical mastitis (Davis et al., 2004; Sundekilde et al., 2013; Luangwilai et al., 2021; Zhu et al., 2021; Bobbo et al., 2022; Gammariello et al., 2024). Milk lactate may have different origins: it can be synthesized by microorganisms or by anaerobic cell metabolism under oxygen-deprived conditions following mastitis (Davis et al., 2004), but the most likely source of lactate is glucose utilization by recruited neutrophils (Gammariello et al., 2024). Leaking of blood lactate into milk across the damaged milk-blood barrier is less probable (Gammariello et al., 2024). Indeed, serum lactate concentration has been reported to be similar in SCM and healthy cows (Dervishi et al., 2017), and the extent of milk lactate increase seems to depend primarily on the SCC rather than the presence of specific infective microorganisms within the infected glands (Davis et al., 2004; Gammariello et al., 2024).

In contrast with previous studies, which evidenced lower levels of glutamate in high-SCC milk (Bobbo et al., 2022) and of glutamate and N-Acetyl-glucosamine in clinical mastitis (Zhu et al., 2021), in our study the bin at 2.295 ppm, falling in a portion of the 300 MHz ^{1}H NMR spectrum corresponding to N-acetyl-glucosamine/N-acetyl-carbohydrates signal was higher in SCM cows and in High-CD11b-SCM samples with respect to healthy samples. This observation agrees with the increase in the enzyme N-acetyl-ß-D-glucodaminidase observed in association with increased SCC (Piccinini et al., 2005). Moreover, glutamate was found at higher level in SCM cows. The possible explanation for these differences may reside on the different pathogens involved. As an example, reduction of glutamate may be related to its use by the activated immune system, but glutamate metabolism can be altered by subclinical mastitis caused by Streptococcus agalactiae (Tong et al., 2019). While N-acetylglucosamine can be utilized by specific bacteria strains (e.g.: SigB-deficient pathotype of bovine Staphylococcus aureus isolated from subclinical mastitis) to produce poly-N-acetylglucosamine-based biofilm (Luangwilai et al., 2021).

4.5. Interdependence of mammary quarters

Observing the fraction of B cells and the expression of adhesion molecules by milk neutrophils (CD11b, CD44, and CD62L) in infected and uninfected quarters, (Blagitz et al., 2015) suggested that the immune response is not independent among mammary quarters, but it is influenced by infected neighboring quarters, despite the anatomical structure of the cow's mammary gland suggests the independence among quarters. These authors did not formulate hypotheses about the mechanisms underlying this phenomenon, but they suggested that it could be influenced by the extent of inflammation, the pathogenicity of the bacteria, the amount of affected tissue and the immune response of individual cows. The immune response to mammary infections can elicit systemic effect(s). A systemic response to the intramammary infusion of LPS as a model of clinical mastitis induced several effects on milk, including a modification of metabolome and, notably, all changes produced in milk showed a delay in comparison with those observed in blood plasma (Johnzon et al., 2018). The systemic metabolic profile was altered also in subclinical intramammary infections possibly depending on the etiological agents, which can affect the immune and inflammatory responses, and increased mammary gland permeability (Lisuzzo et al., 2024). It is therefore conceivable that apparently healthy quarters (Low-CD11b) in SCM cows may be directly or indirectly influenced by the infected neighboring quarters, depending on the involved pathogen(s) and severity of inflammation. Likewise, the fact that some samples classified as Low-CD11b-SCM displayed a modified metabolome might have contributed to the better (less ambiguous) separation between Healthy and SCM affected cows.

4.6. Infection phase

Although this work was not designed to study the effect on milk metabolome of the kinetics of the inflammatory response during mastitis, the time interval between the onset of infection (which was undetermined in this study) and milk sample collection is an important factor that may have led to sample misclassification. Thomas et al. (2016) observed that a modification of metabolite concentration peaked at 81 h after the intramammary infusion of *S. uberis* (a model of clinical mastitis), with milk metabolites returning to near pre-challenge levels at 312 h post-infusion, during the resolution phase. Intramammary LPS infusion, which caused a dramatic increase of milk SCC at 24 h post-infusion persisting for 1 week after the challenge, as well, induced visible modifications of milk metabolome between 24- and 72-hours post-infusion (Johnzon et al., 2018). These studies explored the time course of milk metabolites modifications in pooled milk in animals affected by clinical mastitis, but did not examine single mammary quarters. Therefore, metabolome modifications in SCM cows might have followed different kinetics in Low-CD11b and High-CD11b quarters, even in the case of SCM. As above mentioned, some High-CD11b-SCM samples might have been in a late acute inflammation/early resolution phase.

5. Conclusions

This study has the merit to investigate SCM combining several methodological approaches and to highlight the importance of investigating the biological changes occurring within individual mammary quarters. In particular, the metabonomic approach allowed the classification of healthy and SCM-affected animals with a good accuracy, confirming that increased SCC is associated with modifications of milk metabolome. The classification was less accurate when the mammary quarters were classified in three groups based on the fraction of CD11b positive cells, even though major variations of metabolite content were observed in High-CD11b-SCM samples.

Observations herein reported suggest that in mastitis-affected cows also the healthy mammary quarters can display an altered metabolome, and that the infected quarters can influence the biology of the healthy ones. These findings support the hypothesis of an interdependence of mammary quarters.

This study, however, has some limitations that require further investigations. First, the characterization of the mammary quarters could benefit from the individual SCC data, which may contribute to a better classification. Then, the use of experimental infection models may help control the infection phases, and the CD11b and metabolome dynamics within the individual mammary quarters. Finally, these observations should be extended to a wider population of cows with mammary quarters affected by major pathogens.

List of abbreviations

AOPP	Advanced oxidative stress products
SCC	Somatic Cell Count SCC
SCM	Sub-clinical mastitis
QMS	Quarter milk samples
PLS-DA	Partial least square
sPLS-DA	sparse partial least square
oPLS-DA	Orthogonal projections to latent structures

Declarations

None

Ethical approval

Milk samples used in this work were collected under a program for routinely monitoring mastitis in the dairy farm in accordance with relevant guidelines and regulations, and were not collected for experimental purposes. According to the Italian law for the protection of experimental animals (Law Decree n. 26 issued on 4 March 2014, art. 2), the approval by an ethical committee is not required under the circumstances in which this trial was carried out. The owners of the farm permitted the use of cow milk samples.

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Authors' contributions

GG, AZ & FV conceptualized the study and designed the experiment; FB, PV, EG & MEG look after the collection of milk samples; FB, PV, EG, MEG, AZ & LZ conducted laboratory analyses; VB & LZ analyzed the data; FB, PV, EG, MEG, AZ &LZ drafted the manuscript; EG, GG & FV reviewed and edited the manuscript; FV provided the resources and financial support. All authors contributed to the finalized manuscript, read, and approved the final version.

CRediT authorship contribution statement

Elisa Giaretta: Writing – review & editing, Writing – original draft, Investigation. **Federico Bonsembiante:** Writing – original draft, Methodology. **Valentina Bonfatti:** Data curation. **Lucio Zennaro:** Methodology, Data curation. **Alfonso Zecconi:** Writing – original draft, Investigation, Conceptualization. **Maria Elena Gelain:** Supervision, Methodology, Investigation. **Paola Vanzani:** Writing – original draft, Methodology, Investigation. **Fabio Vianello:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Gianfranco Gabai:** Writing – review & editing, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tvjl.2025.106401.

Availability of data and materials

All data generated and analyzed during this study are included in this published article. Raw data are available from the corresponding author upon reasonable request.

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