JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

pubs.acs.org/JAFC
Article

Subclinical Mastitis from *Streptococcus agalactiae* and *Prototheca* spp. Induces Changes in Milk Peptidome in Holstein Cattle

Alice Vanzin, Cinzia Franchin, Giorgio Arrigoni,* Ilaria Battisti, Antonio Masi, Andrea Squartini, Vittoria Bisutti, Diana Giannuzzi, Luigi Gallo, Alessio Cecchinato, and Sara Pegolo

Cite This: *J. Agric. Food Chem.* 2023, 71, 16827−16839

ABSTRACT: Early detection of bovine subclinical mastitis may improve treatment strategies and reduce the use of antibiotics. Herein, individual milk samples from Holstein cows affected by subclinical mastitis induced by *S. agalactiae* and *Prototheca* spp. were analyzed by untargeted and targeted mass spectrometry approaches to assess changes in their peptidome profiles and identify new potential biomarkers of the pathological condition. Results showed a higher amount of peptides in milk positive on the bacteriological examination when compared with the negative control. However, the different pathogens seemed not to trigger specific effects on the milk peptidome. The peptides that best distinguish positive from negative samples are mainly derived from the most abundant milk proteins, especially from β- and α_{s1}-casein, but also include the antimicrobial peptide casecidin 17. These results provide new insights into the physiopathology of mastitis. Upon further validation, the panel of potential discriminant peptides could help the development of new diagnostic and therapeutic tools.

KEYWORDS: *udder health, Streptococcus agalactiae, Prototheca spp., milk endogenous peptides, mass spectrometry*

INTRODUCTION

Mastitis control is one of the greatest challenges in the dairy industry. In fact, it is well-known that this disease has a negative impact on the sector as it causes reductions in milk quality and quantity,[1,2] resulting in huge economic losses.[3] Moreover, mastitis, in its clinical form, affects animal welfare and it is the main cause of antibiotics treatments in dairy cows.[4] However, the subclinical form of mastitis represents the biggest issue: the animals do not show visible signs of disease, even though an inflammatory process or infection of the udder is ongoing. Besides deterioration in the milk quality, affected animals act as a reservoir of microorganisms that can easily infect other animals within the herd.[5]

Most intramammary infections (IMI) have bacterial origin, but other possible etiological agents include viruses, yeasts, and yeast-like algae of the genus *Prototheca*.[6] Specifically, the incidence of this latter microorganism has steadily increased,[7] and around 11% of Italian dairy herds show currently the presence of this pathogen.[8]

Diagnosis of *Prototheca* spp. infection is difficult and can be often mistaken or missed[9] as this pathogen is able to evade host immunity by surviving macrophage phagocytosis. This, combined with the lack of effective treatments, makes *Prototheca*-caused mastitis a major problem in dairy cattle farming.

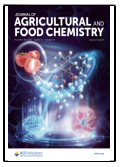
Several studies reported a worsening effect of IMI on milk quality,[2,10] which is directly linked to the composition and activity of somatic cells.[11] These cells are directly involved in the immune response: macrophages and neutrophils are the first line of defense against invading microbes[5,12] through phagocytosis and secretion of compounds such as cytokines, chemokines and proteases.[13] Accordingly, proteolytic activity is reported to be higher in infected than in healthy milk;[14,15] enzymes like plasmin, cathepsin B and D, and elastase are indeed present in higher rates in mastitic milk.[16−19] In addition to host immune cells, invading microbes also release proteases that are involved in protein degradation.[20,21]

The rapid advances in technologies and bioinformatics tools and their increased accessibility have led to a great spread of omics analyses in the animal science field. Specifically, besides genomics and transcriptomics, several studies have been conducted on milk proteome and peptidome alteration in the presence of mastitis in both its clinical and subclinical form. For this purpose, different technologies such as two-dimensional gel electrophoresis followed by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)[13,15,21−29] have been exploited.

Different invading microorganisms can trigger a diverse mammary gland immune response which can be reflected also on specific milk proteome and/or peptidome signatures.[28,30]

In this regard, few information is available about the milk endogenous peptidome upon natural infection from different bacteria.[21] In addition, the effects of other mastitis-causing pathogens such as algae have never been investigated. Therefore, the aim of this study was to evaluate the alteration in the endogenous milk peptidome upon natural subclinical IMI from

Received: May 9, 2023
Revised: August 7, 2023
Accepted: August 8, 2023
Published: October 27, 2023



https://doi.org/10.1021/acs.jafc.3c03065
J. Agric. Food Chem. 2023, 71, 16827−16839

Streptococcus agalactiae (*S. agalactiae*) and *Prototheca* spp. in Holstein Friesian cows. To this end, untargeted and targeted MS approaches were used and combined to identify potential diagnostic milk biomarkers of subclinical pathological conditions and/or pathogen-specific effects.

MATERIALS AND METHODS

Experimental Design and Samples Collection. This study was part of the LATSAN project, which aims to develop innovative tools to study udder health and to improve the nutritional and technological quality of bovine milk, and the AGRITECH project, Spoke 1, task 1.2.2. The research was 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).

Based on a survey on the prevalence of *S. agalactiae* and *Prototheca* spp. conducted by the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe, Legnaro, PD, Italy) in the Veneto region, one herd of 450 lactating Holstein cows located in the province of Verona in northern Italy was selected. The cows were housed in free stalls and fed total mixed rations based mainly on corn silage, sorghum silage, and concentrates. Drinking water was available in automatic water bowls and milking was carried out twice a day.[31] Further details about management and farming conditions are reported in Pegolo et al.[2] Since subclinical conditions can be associated with a low-grade inflammation,[32] the definition of the experimental groups was based only on the results of the bacteriological test, regardless of the somatic cell count (SCC). In detail, the criteria for animals' enrollment in the study were (i) absence of clinical signs of infection or other diseases (e.g., hepatic lipidosis, ketosis, abscesses, laminitis); (ii) no antibiotic treatment or anti-inflammatory medications; and (iii) animals selected as negative controls had no previous history of mastitis. Based on these criteria, bacteriological screening (T0) was carried out on composite milk samples collected by sterile manual milking from 188 animals in accordance with the National Mastitis Council guidelines[33] to identify healthy cows and cows having subclinical IMI from *S. agalactiae* or *Prototheca* spp. Animals with coinfection were excluded from the trial. To avoid potential confounding effects with postpartum metabolic diseases, we selected only multiparous cows and cows having >120 days in milk (DIM). Three groups of animals were identified: negative (Neg, n = 15), positive for *S. agalactiae* (Sa+, n = 12), and positive for *Prototheca* spp. (P+, n = 11). Fifteen days later (T1), the animals belonging to these groups were resampled to confirm the bacteriological results of T0. A 50 mL aliquot, supplemented with the antimicrobial Bronopol 2-bromo-2-nitro-1,3-propanediol (Merck, Darmstadt, Germany), was also collected for the analysis of milk composition and quality traits. Aliquots were kept at 4 °C until the transfer at the IZSVe (Legnaro, PD, Italy) and Milk Quality Laboratory of the Breeders Association of the Veneto Region (ARAV, Padova, Italy), respectively. For proteomic analyses, an additional 50 mL aliquot was taken from individual animals. Aliquots were kept at 4 °C and brought to the Milk Laboratory of the DAFNAE Department (University of Padova). They were immediately centrifuged at 2500*g* for 20 min at 4 °C and the resulting fat layer was removed; the samples were then stored at −80 °C until use. The graphical representation of the experimental design and sample collection is reported in Figure 1.

Microbiological Analysis, Milk Composition, and Udder Health Traits. Microbiological analyses were performed at the IZSVe. After aseptic collection, samples were brought to the laboratory where they were frozen and processed within the following 3 days. The procedure is explained in detail in Pegolo et al.[34]

Analyses of milk composition, SCC, and differential somatic cell count (DSCC), which represents the combined percentage of lymphocytes and polymorphonuclear neutrophils, were run at ARAV laboratory within 24 h of sampling. Milk composition (fat, protein, casein, lactose, and urea content) was determined with the FT6000 Milkoscan infrared analyzer (Foss A/S, Hillerød, Denmark). The udder health traits SCC and DSCC were measured with the FossomaticTM 7 DC analyzer (Foss A/S, Hillerød, Denmark); in order to achieve a normal distribution, SCC were log-transformed to Somatic Cell Score (SCS).[35] Nonparametric Kruskal−Wallis test, followed by a posthoc Dunn test with Benjamini and Hochberg corrections for multiple testing, was carried out to test the effect of the bacteriological status (Negative, Sa+ and P+) in milk yield, milk composition, and udder health traits.

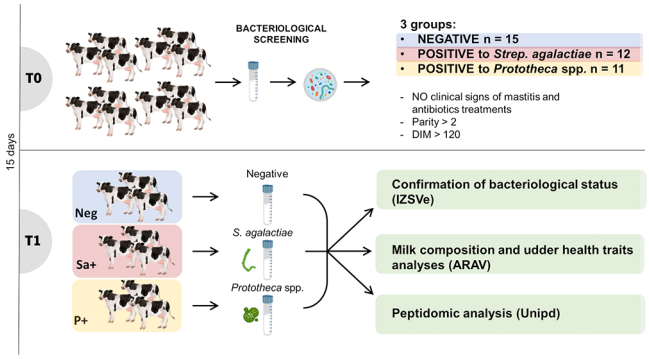


Figure 1. Graphical representation of the experimental design and samples collection.

Samples Preparation and Extraction of Milk Endogenous Peptides. A solution containing 0.1 M BisTris buffer, 6 M GdnHCl, 5.37 mM sodium citrate, and 19.5 mM dl-Dithiothreitol (Sigma-Aldrich, St. Louis, MO) was added to skimmed milk in a 1:1 ratio (v:v) to a total volume of 1 mL; samples were mixed by inversion six times, vortexed 10 s, incubated 1 h at room temperature, and centrifuged at 13,000*g* for 10 min. Avoiding the fat layer, 900 μL of this mixture was filtered through a 0.45 μM filter (GVS Filter Technologies, Bologna, Italy).

Endogenous peptides were collected using the Filter-Aided Sample Preparation (FASP) method.[36] Briefly, 180 μL of the filtered mixture were mixed with 20 μL of a solution A (8 M urea, 100 mM Tris−HCl, pH 8.5). The final volume of 200 μL was added into a centrifugal unit with a molecular weight cutoff of 10 kDa (Vivacon 500, Sartorius, Geottingen, Germany) and centrifuged at 18,600*g* for 15 min. The flow-through was collected in a new 2 mL tube. The centrifugal unit was washed with 200 μL of solution A, centrifuged at 18,600*g* for 15 min and the flow-through was collected in the 2 mL tube. The washing steps were repeated twice, followed by another 10 min centrifugation performed to collect all the residual liquid. All flow-through fractions, containing the endogenous peptides with a MW < 10 kDa were pooled, diluted to a final volume of 1 mL with trifluoroacetic acid (TFA) 0.1%, and pH was adjusted to 3 using TFA 10%. Peptides were desalted with C18 cartridges (Sep-Pak C18, Waters Corp, Milford, MA) according to manufacturer's instructions. Finally, samples were dried under vacuum and stored at −20 °C until instrumental analysis.

MALDI-TOF MS Analyses. Dry samples were suspended in 20 μL of TFA 0.1%. A solution of α-cyano-4-hydroxycinnamic acid (HCCA, 5 mg/mL in 70% acetonitrile/30% TFA 0.1%) was mixed with each sample in a 1:1 ratio (v:v), and 1 μL of this mixture was spotted five times on a stainless-steel MALDI plate and air-dried at room temperature. The analysis was performed in positive, reflectron mode, using a 4800 MALDI-TOF/TOF mass analyzer (Sciex, Framingham, MA) with the following settings: laser intensity of 6000 (arbitrary units), 1500 shots for each spot to be averaged (50 shots acquired in 30 randomly selected different positions), and m/z range 800−4000. Accelerating voltage was set at 20 kV and a delay extraction of 400 ns was selected. Acquisition of all MS spectra (38 samples × 5 replicates) was conducted with a fully automatic process.

MALDI-TOF Data Processing and Statistical Analysis. MALDI-TOF spectra were processed using the MALDIquant[37] and MALDIrppa[38] R packages fitting the pipeline proposed by Gibb and Strimmer;[39] raw spectra were acquired in t2d format and converted in mzXML using the t2d converter tool (freely available at pepchem.org).[40] The mzXML spectra were imported in an R environment. First, data were transformed using the square root transformation and

https://doi.org/10.1021/acs.jafc.3c03065
J. Agric. Food Chem. 2023, 71, 16827−16839

smoothed to reduce the variation of noise with the Savitzky−Golay filter, and then the baseline correction was applied with the TopHat method to remove the background noise due to the matrix effect. Subsequently, a calibration based on the Total Ion Current (TIC) was performed to enable an intensity comparison among different spectra, which were then aligned to match peaks having the same mass. Since signal intensities were comparable, an average spectrum from five technical replicates was created for each biological sample. As a following step, the detectPeaks function was used to identify potential features and reduce the data dimension with the subsequent parameters: Super Smoother method, Signal-to-Noise Ratio (SNR) of 1.5, peak binning with a tolerance of 0.002. Once the features were identified, those that were not present in at least 70% of the samples within each group (Neg, Sa+, P+) were filtered out, and their values were set to zero. An additional matrix was also created: no filter was applied, and all the detected signals higher than the settled background noise were considered. The unfiltered intensity matrix was visualized using a heatmap based on Euclidean distance among samples obtained with the package NMF[41] implemented in the R environment. The filtered intensity matrix was subjected to multivariate analyses. Data were first scaled and visualized using a Principal Component Analysis (PCA) with the R package FactomineR.[42] A Partial Least Square Discriminant Analyses (PLS-DA) was then carried out with the R package mixOmix,[43] in order to select a subset of variables able to discriminate among the categories: positive (Sa+ and P+) vs Neg, Sa+ vs Neg, P+ vs Neg, Sa+ vs P+. To evaluate the PLS-DA performances and choose the number of components to consider, we used 50 times repeated 3-fold cross-validation. Features selection was performed according to a Variable Importance in the Projection (VIP) > 1.

Reproducibility of MALDI Spectra Acquisitions. The reproducibility of MALDI spectra acquisitions was evaluated using two representative samples, a Neg sample and a Sa+ sample, as internal controls. The extraction of milk endogenous peptides was performed from five aliquots of each sample; each peptide mixture was spotted 10 times on a MALDI plate, and spectra were acquired as previously described. Following the data processing steps detailed above, the coefficient of variation (CV) % between samples and within technical replicates was calculated from the intensity matrix.

Untargeted LC-MS/MS Analysis. With the MALDI-MS analysis, we detected many potentially interesting low-intensity signals that, upon direct fragmentation in the MALDI-TOF/TOF system, did not produce spectra of sufficient quality to allow the determination of the peptide sequence. Therefore, we decided to exploit an untargeted LC-MS/MS approach to obtain information about peptide sequences and detect new potentially discriminant peptides. To limit the number of analyses and the biological variability, six pools were created: two for negative (seven + seven samples), two for Sa+ (five + five samples), and two for P+ (five + six samples). Samples that were considered to be of low quality in the previous analysis were excluded and were not used for the pools' creation; samples were pooled to ensure similar average SCC values.

To create the pools, either 0.7 μL (for pools made by 7 samples), 1 μL (for pools made by 5 samples), or 0.8 μL (for the pool made by 6 samples) was taken from each sample and 3% acetonitrile/0.1% formic acid was added to a final volume of 20 μL.

Samples were analyzed with an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA), interfaced to a nano-HPLC Ultimate 3000 (Dionex, Thermo Fisher Scientific, Waltham, MA). A volume of 3 μL of each sample was loaded into a 10 cm pico-frit capillary column (75 μm I.D., 15 μm tip, New Objective) packed in-house with C18 material (Aeris peptide 3.6 um XB-C18, Phenomenex, Torrance, CA). Chromatographic separation of peptides was achieved at 250 nL/min using a linear gradient from 3 to 40% acetonitrile/0.1% formic acid in 90 min. The instrument operated in a data-dependent mode, with a Top10 acquisition method: for each cycle, a full MS scan from 300 to 1700 m/z was acquired at high resolution (60,000) in the Orbitrap, followed by the acquisition of 10 MS/MS spectra of the most intense ions in the linear ion trap. Samples were acquired twice (technical replicates) in a random order and alternated with blanks to avoid column carry-over.

Raw data files were analyzed with the software Proteome Discoverer 1.4 (Thermo Fisher Scientific, Waltham, MA) connected to a Mascot server (v. 2.2.4, Matrix Science) and spectra were searched against the Bovine section of the Uniprot database (v. September 2020, 37,512 sequences) using the following parameters: precursor and fragment tolerance were set to 10 ppm and 0.6 Da, respectively; no enzyme and no fixed modifications were selected, while oxidation of methionine residues was set as variable modification. The precursor area node of Proteome Discoverer was used to integrate the area under the curve for each identified peptide. The algorithm Percolator (v. 2.04) was used to create a decoy database by shuffling protein sequences (Käll et al.[44]) to assess the false discovery rate (FDR) and results were filtered to take into considerations only proteins identified with at least two unique peptides and with a FDR < 0.01, both at the peptide and protein levels. Proteins were grouped into families according to the principle of maximum parsimony. Results were then exported as an Excel spreadsheet for further elaborations.

Data acquired were first used to assign (based on the m/z values in cases where there was no ambiguity) the sequence and protein of origin to the peptides which were identified as discriminant with the MALDI-TOF analysis. Additionally, other peptides not detected with the MALDI-TOF and potentially typical of the various categories Neg, Sa+, P+, and Positive (Sa+ plus P+) were selected from the data. In detail, peptides were considered present in a category if they were detected in at least three out of the four acquisitions (two technical replicates for each of the two pools). Peptides were considered absent for a group if they did not appear in any of the four acquisitions. Peptides considered as preset in Sa+ and P+ and totally absent in the Negative were considered peculiar to the positive group (Sa+ plus P+). The intensities associated with each peptide were averaged within the category and the $\log_2$ Fold Change (FC) was calculated. Finally, a list of candidate peptides potentially able to discriminate among the different groups was created.

Targeted LC-QqQ-MS/MS analysis. To mitigate the risk of possible mismatches between the masses obtained by MALDI and LC-MS/MS data, the selected peptides were validated with an independent MS-based targeted approach. Dry samples previously resuspended in 20 μL of TFA 0.1% were diluted in 3% acetonitrile with a 1:1 ratio, and then 1 μL of each sample was taken to create a pool to use as quality control (QC). The analysis was carried out using a triple quadrupole (TSQ Quantiva, Thermo Fisher Scientific, Waltham, MA) coupled to an UHPLC Ultimate 3000 (Dionex, Thermo Fisher Scientific, Waltham, MA). Peptides were separated by reverse-phase chromatography with a C18 column (Brownlee SPP Peptide ES C18, 100 mm × 2.1 mm i.d., 2.7 μm particle size, PerkinElmer, Waltham, MA) with a 45 min gradient and an injection volume of 4 μL. The detailed instrument parameters are reported in Aita et al.[45] A total of 35 individual samples were randomly analyzed in 5 days, alternated with two pool injections per day as QC. The SRM transitions (at least five for each peptide) were initially chosen based on the manual scrutiny of MS/MS spectra acquired in the untargeted analysis and then experimentally optimized by determining the best collision energy and S-lens values. Raw MS data were analyzed with Skyline MS software v. 21.2.0.425[46] and a relative quantification of each peptide was obtained by using the area of the most intense transition. To assess the repeatability of the analysis, the quantitative data obtained for each peptide in the QC samples were averaged within the day, and the coefficients of variation between the different dates were calculated. Significant differences among experimental groups were assessed by applying the nonparametric Kruskal−Wallis test, followed by a posthoc Dunn test with Benjamini and Hochberg correction for multiple testing. In addition, positive (Sa+ plus P+) and negative samples were compared with a nonparametric Mann−Whitney test and $\log_2$ FC were calculated. Statistical analyses were performed using the R package Stats.[47]

All LC-MS/MS data (from untargeted and targeted analysis) have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD043426.

https://doi.org/10.1021/acs.jafc.3c03065
J. Agric. Food Chem. 2023, 71, 16827−16839

RESULTS

Animals and Data. The final group of 38 animals selected for this study had an average milk yield of 27.13 ± 8.7 kg/day. The bacteriological status had a significantly worsening effect on milk production ($P < 0.01$): both groups of positive animals (Sa + and P+) had a lower milk yield compared to the negative ones (22.95 ± 7.66 kg/day for Sa+, 24.24 ± 9.28 kg/day for P+ and 32.59 ± 6.25 kg/day for Neg; Table 1). No difference in milk production was detected when animals infected by *S. agalactiae* were compared with animals infected by *Prototheca* spp. Among the composition traits, milk lactose percentage was the only one showing significant differences among experimental groups ($P < 0.05$; Table 1), with a lower percentage in the P+ group when compared with the negative one (4.34 ± 0.38 vs 4.74 ± 0.32). Udder health indicators (SCS and DSCC, reported in Table 1) showed a significant difference in terms of SCS ($P < 0.001$) between negative and positive samples. We observed higher SCS in positive samples (6.98 ± 1.99 and 6.58 ± 1.46 for Sa+ and P+ respectively) compared to the negative (2.42 ± 0.96), but no significant difference between pathogens.

Mass Spectrometry Analysis Workflow. An aliquot of milk from each individual animal was used for the peptidomic profiling: endogenous peptides were isolated using filters with a 10 kDa cutoff and analyzed combining different mass spectrometry techniques. Initially, an untargeted MALDI-TOF MS approach was applied in order to select the features that are potentially able to discriminate between the different categories of animals. On the same samples, a LC-MS/MS analysis was performed to identify the peptides, which were then quantified and validated through a targeted LC-MS/MS analysis. The graphical representation of the experimental workflow is reported in Figure 2, while all details regarding the experimental procedures are described in the Methods section.

Untargeted MALDI-TOF MS Analyses and Features Selection. The untargeted MALDI-TOF analyses of endogenous peptides were performed on all samples. Good repeatability was found between the technical replicates (CV = 19%) and moderate between different extractions from the same sample (CV = 29%). Three spectra were excluded because they were characterized by peaks not distinguishable from the background noise (n = 1 Neg, 2 Sa+). The final number of samples retained for further analyses was 35.

Data processing produced two intensity matrices with peptides expressed as m/z values (n = 642 and n = 68 for unfiltered and filtered matrices, respectively) reported as columns and the intensity values obtained for each sample reported as rows.

Figure 3 displays the total number of features (supposedly corresponding to endogenous peptides) obtained in each sample (overall mean of 86.36 peptides for Neg, 114.6 peptides for Sa+ and 107.36 peptides for P+). Bacteriological status shows a significant effect ($P < 0.05$) on the number of individual peptides, with *S. agalactiae* and *Prototheca* spp. positive samples characterized by a higher number of features with respect to the negative samples ($P < 0.05$ and $P < 0.1$, respectively).

The heatmap for the 200 most abundant features (unfiltered data) is displayed in Figure 4. Two large clusters were identified, which included most of the positive and negative samples, respectively. On the other hand, no clear separation was observed between Sa+ and P+ groups.

The filtered intensity matrix (features present in more than 70% of the samples of one group) included 68 putative peptides

Table 1. Milk Yield, Standard Composition, and Udder Health Traits of Cows[a]

item	negative					Sa+					P+					P-value[b]
	N	mean	SD	Q1	Q99	N	mean	SD	Q1	Q99	N	mean	SD	Q1	Q99	
milk yield, kg/day	15	32.59[a]	6.25	22.60	44.83	12	22.95[b]	7.66	10.02	36.52	11	24.24[b]	9.28	12.53	40.96	0.004**
milk composition																
fat, %	15	2.13	0.76	1.07	3.44	12	2.18	0.70	1.41	3.31	11	2.02	0.82	1.04	3.31	0.761
protein, %	15	3.49	0.22	3.00	3.90	12	3.48	0.30	3.14	4.20	11	3.52	0.31	3.08	3.96	0.687
casein, %	15	2.77	0.20	2.34	3.11	12	2.71	0.28	2.36	3.36	11	2.72	0.27	2.35	3.11	0.445
lactose, %	15	4.74[a]	0.32	4.04	5.20	12	4.48[a,b]	0.48	3.46	4.92	11	4.34[b]	0.38	3.69	4.85	0.041*
udder health traits																
SCS, units[c]	15	2.42[a]	0.96	0.05	3.47	12	6.98[b]	1.99	4.05	9.65	11	6.58[b]	1.46	3.47	8.37	<0.001***
DSCC, %	12	58.89	12.43	45.22	77.16	6	65.05	6.71	60.17	76.95	4	58.15	15.18	39.34	73.01	0.631
pH	15	6.49	0.08	6.37	6.63	12	6.43	0.07	6.31	6.54	11	6.45	0.09	6.29	6.59	0.2043
conductivity, mS/cm	15	9.26	0.86	8.04	11.23	12	1001.94	143.12	866.32	1284.91	11	1042.05	115.09	877.67	1194.77	0.071

[a](a, b) Different letters indicate significant differences between groups (post hoc Dunn test with a Benjamini and Hochberg correction). Sa+, Streptococcus agalactiae positive group; P+, *Prototheca* spp. positive group; SCS, somatic cell score; DSCC, differential somatic cells count. [b]Nonparametric Kruskal–Wallis test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. [c]A SCS = 2 corresponds to SCC = 100,000 cells/mL, SCS = 6 corresponds to SCC = 800,000 cells/mL.

https://doi.org/10.1021/acs.jafc.3c03065
J. Agric. Food Chem. 2023, 71, 16827−16839

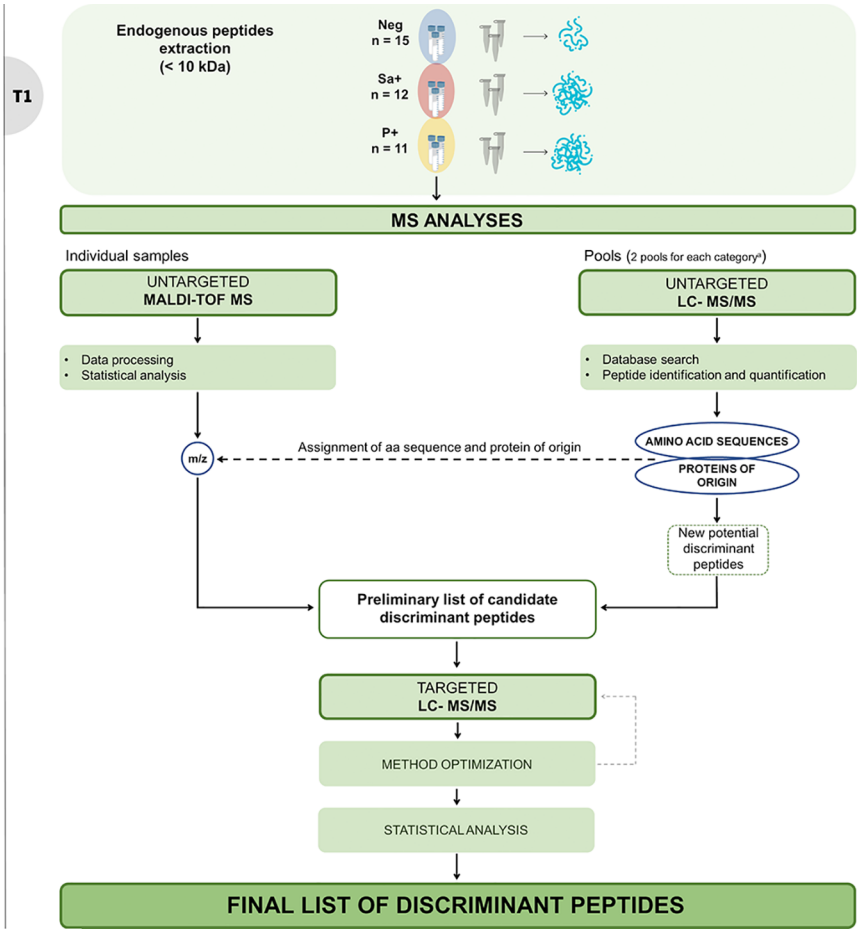


Figure 2. Graphical representation of the experimental workflow. Neg, animals negative on bacteriological examination; Sa+, animals positive for *Streptococcus agalactiae*; P+, animals positive for *Prototheca* spp. [a] Two technical replicates for each of the two pools.

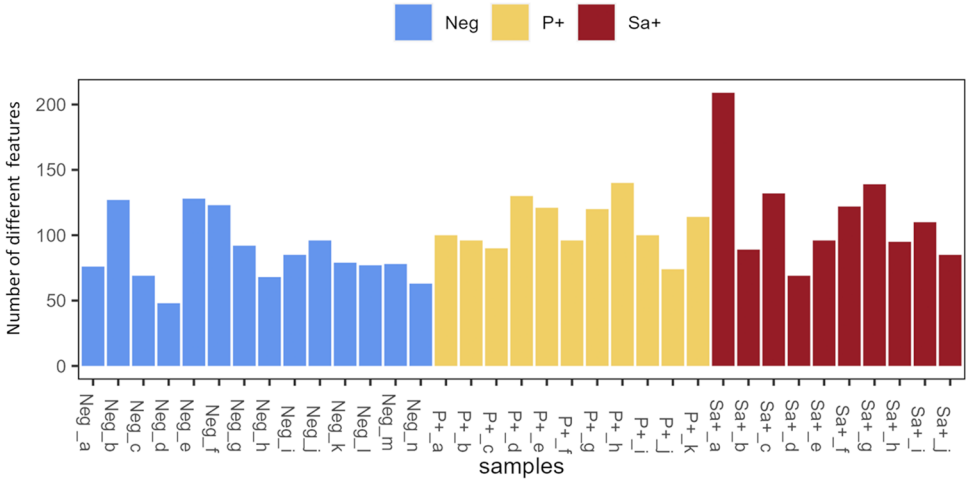


Figure 3. Barplot showing the total number of features identified in each sample by MALDI-TOF MS analysis. *Y*-axis shows the number of features. *X*-axis shows all the samples: n = 14 Neg (blue), n = 11 P+ (yellow), n = 10 Sa+ (red). Low quality samples (n = 3) are not displayed. Neg, animals negative on bacteriological examination; Sa+, animals positive for *Streptococcus agalactiae*; P+, animals positive for *Prototheca* spp.

(n = 38 in Neg, n = 63 in Sa+, and n = 48 in P+). The PCA was performed on this matrix and the result is displayed in Figure 5, which evidences a separation of samples according to the infection status (positive and negative) and according to the specific pathogen (Sa+ and P+); (PC1 = 34.86%, PC2 = 18.73%).

The complete list of peptides with VIP > 1 identified by PLS-DA analysis is reported in Supplementary Table S1. In total, 48 unique peptides with VIP > 1 were able to discriminate the groups in at least one pairwise comparison (n = 27 Neg vs Pos, n = 35 Neg vs Sa+, n = 35 Neg vs P+ and n = 23 Sa+ vs P+).

Peptides Identification by Untargeted LC-MS/MS Analysis. The untargeted MALDI-TOF MS analysis provided

https://doi.org/10.1021/acs.jafc.3c03065
J. Agric. Food Chem. 2023, 71, 16827−16839

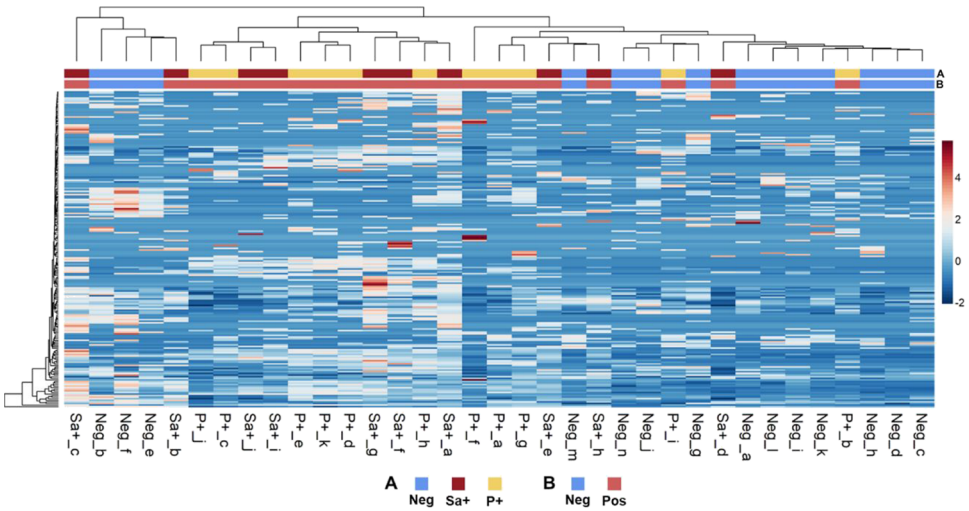


Figure 4. Heatmap with *Z*-score clustering based on Euclidean distance. Each row represents a feature (m/z), and each column represents a sample. Bars above the plot indicate the samples' categories: A− negative, *S. agalactiae* (Sa+), *Prototheca* spp. (P+); B− negative and positive (Sa+ plus P+). Color scale is based on signal intensity deviation from the mean (*Z*-score) with blue for less intense and red for more intense.

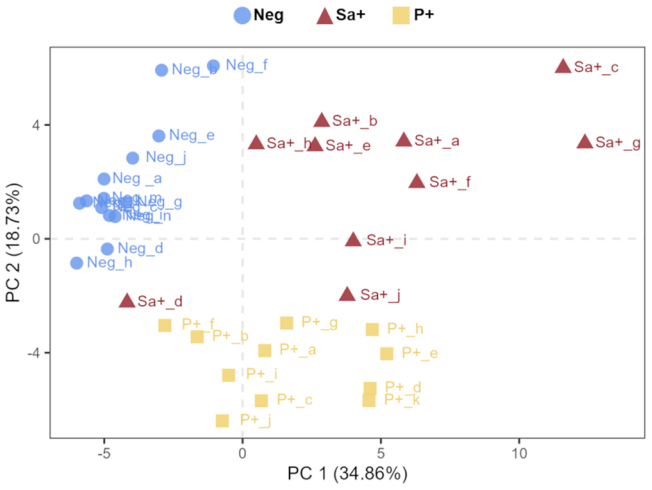


Figure 5. PCA plot of milk samples according to data obtained from the MALDI-TOF MS analysis. Each dot represents a sample. Principal component 1 (PC1) explains the 34.86% of the variance between samples and it is shown in the *x*-axis. Principal component 2 (PC2) explains the 18.73% of the variance between samples and it is shown in the *y*-axis. Neg, animals negative on bacteriological examination; Sa+, animals positive for *Streptococcus agalactiae*; P+, animals positive for Prototheca spp.

a milk peptidome fingerprint, from which m/z values and signal intensities were obtained, but without information about peptides' sequences and protein of origin. In addition, small and low abundance peptides as well as peptides poorly ionized by the MALDI source may have been missed. The low intensity of several signals did not allow a direct fragmentation within the MALDI-TOF/TOF system; therefore, we decided to analyze the same samples using a more sensitive untargeted LC-MS/MS approach. To limit the number of analyses, samples were pooled as described in the Methods sections and a data-dependent acquisition LC-MS/MS approach was undertaken. All identified peptides are listed in Supplementary Table S2. By exploiting the high mass accuracy of the Orbitrap and TOF analyzers, we were able to match the molecular weight of the identified peptides with the m/z values obtained from MALDI-TOF MS analysis and therefore assign a sequence to the potentially discriminating peptides selected through the PLS-DA. In total, out of the 48 selected VIP, 23 were identified without ambiguity, which derived from the most abundant milk proteins: α_{s1}-casein, α_{s2}-casein, β-casein, and β-lactoglobulin (Table 2).

The remaining 25 features selected by the PLS-DA could not be assigned to any specific peptide sequence because the respective m/z values were not detected in the LC-MS/MS analysis or corresponded to more than one sequence.

However, additional peptides not previously detected by MALDI-TOF MS analysis were also identified. In accordance with the previous observation based on the number of features, positive samples contained more peptides than the negative ones: 36 identified peptides in the negative pools versus 198 in the Sa+ pools and 179 in the P+ pools, on average (Supplementary Table S2). In total, we selected 25 new peptides derived from nine different proteins: α_{s1}-casein, α_{s2}-casein, κ-casein, β-casein, β-lactoglobulin, Butyrophilin subfamily 1 member A1, Glycosylation-dependent cell adhesion molecule 1, Glycoprotein 2, and Lactoperoxidase (Table 3). Five peptides were present in all samples with a $\log_2$ FC > 1, regardless of the bacteriological status between positive and negative group; eight peptides were present only in the positive group; nine and three peptides were exclusive of Sa+ and P+ group, respectively. No peptide was exclusive of the negative group.

The list of the 23 previously identified peptides by MALDI TOF analyses and the 25 new peptides identified and selected from the LC- MS/MS analyses made up a comprehensive panel of features apparently capable of distinguishing the different experimental groups. Four of these peptides were present both in the normal form and with an oxidized methionine, and they were all included in the final panel, for a total of 52 peptides.

Quantitative Differences of Selected Discriminant Peptides. To validate the results obtained with the untargeted approaches and quantify the differences in abundance of peptides among experimental groups, all individual samples were analyzed by a LC-QqQ-MS/MS selected reaction monitoring (SRM) approach, targeting the 52 selected peptides. After the optimization of a scheduled method, we were able to simultaneously monitor 40 peptides (the list of all transitions is reported in Supplementary Table S3). After testing the

https://doi.org/10.1021/acs.jafc.3c03065
J. Agric. Food Chem. 2023, 71, 16827−16839

Table 2. List of Peptides Selected through PLS-DA and Identified by the Untargeted LC-MS/MS Analysis

m/z	peptide sequence	protein identity[a]
994.48	EDVPSERY	α_{s1}-CN (99−106)
1140.69	RPKHPIKHQ	α_{s1}-CN (16−24)
1232.78	GYLEQLLRLK	α_{s1}-CN (108−117)
1409.81	FYPELFRQFY	α_{s1}-CN (160−169)
1523.96	LSLSQSKVLPVPQK	β-CN (178−191)
1637.05	AMKPWIQPKTKVIP	α_{s2}-CN (204−217)
1782.08	YQEPVLGPVRGPFPII	β-CN (208−223)
1881.16	YQEPVLGPVRGPFPIIV	β-CN (208−224)
1895.17	LYQEPVLGPVRGPFPII	β-CN (207−223)
1994.25	LYQEPVLGPVRGPFPIIV	β-CN (207−224)
2008.27	LLYQEPVLGPVRGPFPII	β-CN (206−223)
2107.35	LLYQEPVLGPVRGPFPIIV	β-CN (206−224)
2265.35	LIVTQTMKGLDIQKVAGTWY	LGB (17−36)
2325.45	AFLLYQEPVLGPVRGPFPIIV	β-CN (204−224)
2347.45	AmKPWIQPKTKVIPYVRYL[b]	α_{s2}-CN (204−222)
2460.52	RPKHPIKHQGLPQEVLNENLL	α_{s1}-CN (16−36)
2616.63	RPKHPIKHQGLPQEVLNENLLR	α_{s1}-CN (16−37)
2699.61	LVYPFPGPIPNSLPQNIPPLTQTPV	β-CN (73−97)
2794.73	MPIQAFLLYQEPVLGPVRGPFPIIV	β-CN (200−224)
3396.03	AVPYPQRDMPIQAFLLYQEPVLGPVRGPFP	β-CN (192−221)
3622.23	AVPYPQRDMPIQAFLLYQEPVLGPVRGPFPII	β-CN (192−223)
3646.1	EGIHAQQKEPMIGVNQELAYFYPELFRQFY	α_{s1}-CN (140−169)
3756.12	YVPLGTQYTDAPSFSDIPNPIGSENSEKTTMPLW	α_{s1}-CN (181−214)

[a] α_{s1}-CN, α_{s1}-casein; α_{s2}-CN, α_{s2}-casein; β-CN, β-casein; LGB, β-lactoglobulin. The position in mature bovine amino acid sequence is reported in brackets. [b] Presence of the oxidated form of methionine.

Table 3. List of Selected Additional Peptides Discovered from the LC-MS/MS Analysis[a]

m/z	peptide sequence	Protein identity[b]	Group[c]
1584.88	LPQEVLNENLLRF	α_{s1}-CN (26−38)	all
1981.86	FQSEEQQQTEDELQDK	β-CN (48−63)	all
2480.18	KIEKFQSEEQQQTEDELQDK	β-CN (44−63)	all
1494.79	LVEDHIAEGSVAVR	BTN1A1 (97−110)	all
1979.09	SEGVAIDPARVLDLGPITR	GP2 (481−499)	all
2300.14	NAVPITPTLNREQLSTSEENS	α_{s2}-CN (130−150)	Pos
1686.98	LIVTQTMKGLDIQKV	LGB (17−31)	Pos
1276.69	PFPEVFGKEKV	α_{s1}-CN (42−52)	Pos
1290.82	LPLSILKEKHL	GLYCAM1 (83−93)	Pos
2150.19	SSRQPQSQNPKLPLSILKE	GLYCAM1 (72−90)	Pos
1493.81	QGPIVLNPWDQVK	α_{s2}-CN (116−128)	Pos
1500.75	HIQKEDVPSERY	α_{s1}-CN (95−106)	Pos
1918	VPQLEIVPNSAEERLHS	α_{s1}-CN (121−137)	Pos
1438.79	SDKIAKYIPIQY	κ-CN (40−51)	Sa+
1684.68	EGQEQEGEEMAEYR	BTN1A1 (79−92)	Sa+
2383.16	NMAINPSKENLCSTFCKEVVR	α_{s2}-CN (40−60)	Sa+
1143.56	EIVPNSAEER	α_{s1}-CN (125−134)	Sa+
1611.78	EIVPNSAEERLHSM	α_{s1}-CN (125−138)	Sa+
1476.77	IQKEDVPSERYL	α_{s1}-CN (96−107)	Sa+
1696.86	IQKEDVPSERYLGY	α_{s1}-CN (96−109)	Sa+
1219.64	VAPFPEVFGKE	α_{s1}-CN (40−50)	Sa+
3602.99	VLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPV	β-CN (185−216)	Sa+
1929.94	TLSSEAPTTQQLSEYFK	LPO (91−107)	P+
1784.93	IESPPEINTVQVTSTAV	κ-CN (174−190)	P+
2116.92	VFVSREGQEQEGEEMAEY	BTN1A1 (74−91)	P+

[a] Peptides were selected based on different criteria: $\log_2$ FC > 1 between groups or present in the positive animals and not present in the negative animals. All, present in every group; Pos, present in groups of animals positive for *Streptococcus agalactiae* and *Prototheca* spp.; Sa+, present only in animals positive for *Streptococcus agalactiae*; P+, present only in animals positive for *Prototheca* spp. [b] α_{s1}-CN, α_{s1}-casein; α_{s2}-CN, α_{s2}-casein; κ -CN, κ-casein; β-CN, β-casein; LGB, β-lactoglobulin; BTN1A1, Butyrophilin subfamily 1 member A1; GP2, Glycoprotein 2; LPO, Lactoperoxidase; GLYCAM1, Glycosylation-dependent cell adhesion molecule 1. The position in mature bovine amino acid sequence is reported in brackets. [c] Sa+, animals positive for *Streptococcus agalactiae*; P+, animals positive for *Prototheca* spp.

https://doi.org/10.1021/acs.jafc.3c03065
J. Agric. Food Chem. 2023, 71, 16827−16839

Table 4. Results of Kruskal–Wallis and Dunn's Post Hoc Test on Peptides Subjected to LC-QqQ-MS/MS SRM Analysis[a,b]

peptide sequence	protein identity[c]	Sa+ vs Neg		P+ vs Neg		CV %
		P-value[d]	log_2FC	P-value[d]	log_2FC	
RPKHPIKHQ	α_{s1}-CN (16–24)	0.023*	2.65	0.003**	2.40	4.62
HIQKEDVPSERY	α_{s1}-CN (95–106)	<0.001***	4.68	<0.001***	3.75	7.65
EDVPSERY	α_{s1}-CN (99–106)	0.008**	2.53	<0.001***	1.94	4.36
EIVPNSAEER	α_{s1}-CN (125–134)	<0.001***	3.63	0.004**	2.39	5.79
IQKEDVPSERYL	α_{s1}-CN (96–107)	<0.001***	3.42	<0.001***	4.04	8.41
RPKHPIKHQGLPQEVLNENLLR	α_{s1}-CN (16–37)	0.074	1.39	0.0562	1.87	8.99
RPKHPIKHQGLPQEVLNENLL	α_{s1}-CN (16–36)	0.178	1.40	0.031*	1.98	12.85
VPQLEIVPNSAEERLHS	α_{s1}-CN (121–137)	<0.001***	2.72	0.002**	2.11	8.56
PFPEVFGKEKV	α_{s1}-CN (42–52)	<0.001***	3.36	<0.001***	4.52	6.58
VAPFPEVFGKE	α_{s1}-CN (40–50)	0.056	1.40	0.049*	1.08	4.58
GYLEQLLRLK	α_{s1}-CN (108–117)	0.093	1.33	0.063	2.27	15.29
LPQEVLNENLLRF	α_{s1}-CN (26–38)	0.005**	2.05	0.007**	1.87	8.85
FYPELFRQFY	α_{s1}-CN (160–169)	0.018*	6.07	0.003**	7.43	6.59
AMKPWIQPKTKVIP	α_{s2}-CN (204–217)	0.06	2.48	0.002**	3.26	7.65
AmKPWIQPKTKVIPYVRYL	α_{s2}-CN (204–222)	0.28	0.86	0.076	1.11	8.99
NMAINPSKENLCSTFCKEVVR	α_{s2}-CN (40–60)	0.174	1.46	0.014*	2.34	5.24
NmAINPSKENLCSTFCKEVVR	α_{s2}-CN (40–60)	0.391	1.46	0.111	2.34	12.01
QGPIVLNPWDQVK	α_{s2}-CN (116–128)	0.016*	2.84	<0.001***	3.75	12.08
KIEKFQSEEQQQTEDELQDK	β-CN (44–63)	0.005**	1.78	0.003**	1.97	19.56
LSLSQSKVLPVPQK	β-CN (178–191)	<0.001***	4.27	<0.001***	4.41	7.26
YQEPVLGPVRGPFPII	β-CN (208–223)	0.007**	4.04	<0.001***	5.25	8.57
LYQEPVLGPVRGPFPII	β-CN (207–223)	0.006**	4.81	<0.001***	5.42	10.92
YQEPVLGPVRGPFPIIV	β-CN (208–224)	<0.001***	3.31	<0.001***	3.36	11.65
LYQEPVLGPVRGPFPIIV	β-CN (207–224)	<0.001***	3.17	<0.001***	2.90	11.92
LLYQEPVLGPVRGPFPII	β-CN (206–223)	0.014*	4.98	<0.001***	6.15	12.52
LLYQEPVLGPVRGPFPIIV	β-CN (206–224)	<0.001***	4.56	<0.001***	4.61	12.96
SDKIAKYIPIQY	k-CN (40–51)	<0.001***	8.29	<0.001***	8.91	11.82
IESPPEINTVQVTSTAV	k-CN (174–190)	0.022*	1.27	<0.001***	2.65	13.34
LIVTQTMKGLDIQKV	LGB (17–31)	<0.001***	6.64	<0.001***	5.29	9.01
LIVTQTMKGLDIQKVAGTWY	LGB (17–36)	<0.001***	5.93	<0.001**	4.35	15.89
LIVTQTmKGLDIQKVAGTWY	LGB (17–36)	0.002**	5.93	<0.002**	4.35	11.25
LVEDHIAEGSVAVR	BTN1A1 (97–110)	0.005**	0.83	<0.001***	1.11	11.93
SEGVAIDPARVLDLGPITR	GP2 (481–499)	1	0.32	0.752	0.11	8.8
LPLSILKEKHL	GLYCAM1 (83–93)	0.861	0.45	0.76	−0.05	7.93

[a]The CV% (coefficient of variation) refers to the quantitative data obtained in the quality control samples [b]Neg, animals negative on bacteriological examination; Sa+, animals positive for *Streptococcus agalactiae*; P+, animals positive for *Prototheca* spp. [c]α_{s1}-CN = α_{s1}-casein, α_{s2}-CN = α_{s2}-casein, β-CN = κ-casein, β-CN = β-casein, LGB = β-lactoglobulin, BTN1A1 = Butyrophilin subfamily 1 member A1, GLYCAM1 = Glycosylation-dependent cell adhesion molecule 1, and GP2 = Glycoprotein 2. The position in mature bovine amino acid sequence is reported in brackets [d]P-value were adjusted with Benjamini and Hochberg method. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

repeatability of quantitative data, we retained for statistical analyses 34 peptides showing a CV < 20% across all samples.

The associations between the bacteriological status and selected peptides are shown in Table 4. We found that 24 and 28 peptides were more abundant in Sa+ e P+ groups when compared with the negative group ($P < 0.05$). Samples from positive animals (Sa+ and P+) shared 24 peptides mainly derived from α_{s1}-casein and β-casein, followed by κ-casein, β-lactoglobulin, and one single peptides derived from α_{s2}-casein and Butyrophilin subfamily 1 member A1. Four α_{s1}-casein and α_{s2}-casein-derived peptides were different only for P+ vs Neg comparison ($P < 0.05$). No peptide was able to discriminate between Sa+ and P+ groups.

Based on these results, we repeated the analyses by considering positive animals as a single group and comparing this group with the Neg one. We found significant differences in the abundance of 31 peptides (Figure 6a and Supplementary Table S4). In addition to the previous significant peptides, two α_{s1}-casein-derived peptides (RPKHPIKHQGLPQEVLNENLLR and GYLEQLLRLK) and one α_{s2}-casein-derived peptide (AmKPWIQPKTKVIPYVRYL) had gained significance ($P < 0.05$).

Each of these peptides was more abundant in positive samples: the highest log_2 FC (Pos/Neg) were provided from a fragment of κ-casein (SDKIAKYIPIQY; log_2 FC = 8.66; $P < 0.001$), a fragment of α_{s1}-casein (FYPELFRQFY; log_2 FC = 6.94; $P < 0.001$), and a fragment of β-lactoglobulin (LIVTQTMKGLDIQKV; log_2 FC = 6.1; $P < 0.001$). Nineteen peptides had a log_2 FC between 5 and 2, while nine peptides had a log_2 FC < 2. Among the latter, the log_2 FC of a α_{s2}-casein- and a Butyrophilin subfamily 1 member A1-derived peptides were <1 (AmKPWIQPKTKVIPYVRYL, log_2FC = 0.996 and $P < 0.5$; LVEDHIAEGSVAVR, log_2 FC = 0.981 and $P < 0.001$). Only three peptides derived from α_{s2}-casein (NmAINPSKENLCSTFCKEVVR), glycosylation-dependent cell adhesion molecule 1 (LPLSILKEKHL), and Glycoprotein 2 (SEGVAIDPARVLDLGPITR) were not significant when comparing Pos and Neg, confirming the previous pathogen-

https://doi.org/10.1021/acs.jafc.3c03065
J. Agric. Food Chem. 2023, 71, 16827–16839

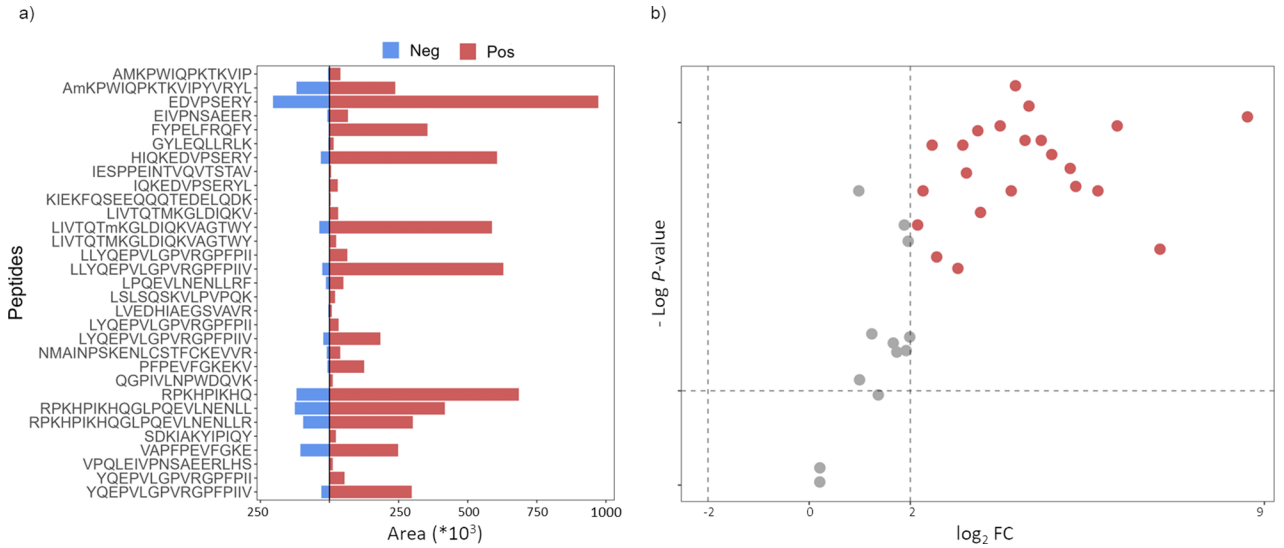


Figure 6. (a) Barplot of peptide content in negative (blue) and positive (red) samples. All the reported peptides were significantly more abundant in the Pos group ($P < 0.05$). (b) Plot of the differential abundant peptides between the negative and positive group. Each dot represents a peptide, and it is plotted according to its $\log_2$ FC in the *x*-axis and the *P*-value obtained from Mann−Whitney test. Red dots represent peptides with a $\log_2$ FC > 2 and $P <$ 0.05. Neg, animals negative on bacteriological examination; Pos, animals positive for *Streptococcus agalactiae* or *Prototheca* spp.

specific statistical analyses. The plot reported in Figure 6b represents with the red dots the peptides that met both conditions of significance ($P < 0.001$) and $\log_2$ FC > 2.

DISCUSSION

In this study, we used a mass-spectrometry-based approach to investigate the differences in the endogenous milk peptidome between healthy dairy cows and animals naturally infected by *S. agalactiae* or *Prototheca* spp. The idea is that finding specific peptidome fingerprints might be helpful not only to increase the biological knowledge on the relationships between host and pathogens and the mammary gland response to infection and inflammation but also to identify potential biomarkers of subclinical pathological conditions. The untargeted MALDI-TOF MS analyses provided a picture of the peptides present in the different samples and the subsequent untargeted LC-MS/MS analysis provided the peptide sequences. A final targeted SMR approach allowed the simultaneous quantification of the 40 selected features in a relatively short time (45 min) which could be useful for screening purposes.

Both the untargeted analyses carried out through the MALDI-TOF MS and the LC-MS/MS approaches evidenced a higher amount of peptides in the infected milk when compared to the healthy one, in agreement with previous studies.[15,27] On the other hand, *S. agalactiae* and *Prototheca* spp. seemed not to trigger pathogen-specific proteolytic effects on the milk peptidome. Mansor et al.[21] also assessed the milk peptidome variation upon different causative agents of infection (i.e., *Staphylococcus aureus* and *Escherichia coli*) but they found a group of peptides statistically able to discriminate the different origin of the clinical mastitis. These different results might be due to differences in the investigated pathogens, number of samples, MS approach, stage, and severity of the disease. In addition, Thomas et al.[48] observed that modifications in proteome and peptidome occur even when the pathogen (i.e., *Streptococcus uberis*) has been eradicated. Therefore, it is reasonable to suppose that the bacteriological status may not always correspond to the peptide profile: a high content of endogenous peptides may be found in samples that have recently resolved the infection, whereas a less rich peptidome may also be found in positive samples in which strong proteolysis has not yet been triggered.

The peptides that best distinguished the positive from negative samples derived from the most abundant milk proteins, i.e., caseins, especially from β- and α_{s1}-casein. Also, these results are in agreement with outcomes of previous research, upon clinical[21,48] and subclinical[15,27] conditions and independently from the causative agents. Three α_{s1}-casein-derived peptides cleaved between amino acids 95−105, which we found significantly increased in positive samples ($\log_2$ FC > 2), correspond (in total or partially) to peptides found in a study conducted by Addis et al.,[27] focused on coagulase-negative staphylococci (CNS)-induced subclinical mastitis. The peptide HIQKEDVPSERY (95−106) was also found to be discriminant by Addis et al.,[27] but with the addition of one amino acid (95−107). Other studies also showed that the abundance of this peptide increases in clinical mastitic milk.[48] Two shorter peptides, IQKEDVPSERYL (96−107) and EDVPSERY (99−106), were also consistent with previous studies[27] except for an additional leucine in the C-terminal of the latter. Moreover, the peptide PFPEVFGKEKV (42−52) ($\log_2$ FC = 4.08 in positive vs negative samples) was also previously detected in the milk of dairy cows infected by different bacteria.[21,27]

Positive samples were also rich in β-casein-derived peptides. Among these, the LSLSQSKVLPVPQK (178−191) peptide, significantly more abundant in the positive samples, has been also included by Addis et al.[27] in the panel of peptides that potentially discriminate subclinical mastitic milk from healthy ones. A further set of seven discriminant peptides came from the C-terminal region LLYQEPVLGPVRGPFPIIV (206−224) of the β-casein. Two of these, LLYQEPVLGPVRGPFPII (206−223) and LYQEPVLGPVRGPFPII (207−223), were also found to be increased by Mansor et al.[21] in mastic milk.

We found few peptides originating from α_{s2}-casein showing higher abundance in the positive samples: in particular, the peptide QGPIVLNPWDQVK (116−128) was also detected by Addis et al.[27] Notably, the peptide with the highest $\log_2$ FC (8.66) belong to the κ-casein SDKIAKYIPIQY (40−51) but it

https://doi.org/10.1021/acs.jafc.3c03065
J. Agric. Food Chem. 2023, 71, 16827−16839

did not match with any of the sequences already reported in the literature, although it is embedded in a much longer peptide (40−69, SDKIAKYIPIQYVLSRYPSYGLNYYQQKPV) described by Addis et al.[27]

Apart from the caseins-derived peptides, in accordance with other studies,[21,27] we also discovered discriminating peptides originated from other proteins, such as β-lactoglobulin, lactoperoxidase, osteopontin, butyrophilin subfamily 1 member A1, glycosylation-dependent cell adhesion molecule 1.

Concerning β-lactoglobulin, we found two peptides LIVTQTMKGLDIQKV (17−31) and LIVTQTMKGLDIQKVAGTWY (17−36) increased in the positive group with respect to the negative group with $\log_2$ FC of 6.1 and 5.2, respectively. The identification of peptides derived from β-lactoglobulin is quite rare: only a few peptides were found from Addis et al.[27] and Mansor et al.,[21] and mainly in healthy milk compared to mastitic milk, where the decrease in complete-protein production resulted in a decrease in peptides produced. Our discordant result may be due to differences in the stage of infection.

Proteases play a key role in the type and number of peptides released in milk during mastitis. The invading microorganisms trigger a host's immune response, resulting in the release of components from blood into milk[49] including a large influx of somatic cells, which is closely linked to the release and increase in activity of several proteases.[17,50,51] However, proteolytic enzymes produced to fight infection lack in specificity[13] so, as we observed in the present study, they can mainly act against the most abundant milk proteins like caseins, releasing a great amount of small caseins-derived peptides. Several studies demonstrated that milk proteins are degraded mainly by plasmin, cathepsin B and D, and elastase.[15,22,52] At low SCC levels, it is plasmin that drives proteolysis,[15,52] but when SCC increases, it leads to a significant and consequential higher presence and activity of the lysosomal proteinase cathepsins B, D and elastases.[15,22] The action of exopeptidases has also been observed in subclinical mastitic milk,[15] which may explain the large number of peptides found in our study which differed only by the presence or absence of one N-terminal or C-terminal amino acid.

A role in the breakdown of milk proteins can also be covered by the microbial extracellular proteases,[49] as reported also by Åkerstedt et al.,[53] which demonstrated the in vitro activity of different strains of *S. agalactiae* on milk especially against α_{s2}-casein. In this research, no differences in milk peptidome were observed between *S. agalactiae* and *Prototheca* spp. This could be due to the fact that proteolysis seems to be mainly driven by endogenous proteases, as suggested by other authors.[27,48] In particular, Thomas et al.[48] showed that, following artificial *S. uberis* infection, the proteome and peptidome of milk still change even when the infection is resolved, supporting the hypothesis that degradation is related more to the immune response than to the etiological agent.

Some authors also found a considerable increase in acute phase proteins[48] after occurrence of mastitis, in addition to the decrease in caseins[34,54,55] and the increase in blood serum proteins.[56] In our study, we did not find any significant peptide derived by proteins such as α-lactalbumin, secretory IgA, or lactoferrin, in line with other studies.[27,52] The different extents of proteolysis that milk proteins undergo upon infection may be due to their different structure. The weaker tertiary structure of caseins may be responsible for the increase in their susceptibility to enzymatic degradation.[57] On the flip side, the more compact structure of α-lactalbumin, lactoferrin, and IgA could protect them from the action of proteolytic enzymes.

Antimicrobial peptides such as cathelicidin have been proposed as mastitis indicators upon *S. agalactiae* infection both in subclinical[26] and clinical forms.[58] In this study, however, we did not detect any significant cathelicidin-derived peptide, in line with other studies on subclinical mastitis.[15,22,27] A possible explanation might be the large presence of casein proteins in milk, which may prevent the detection of less representative proteins or the subclinical condition of the disease. Nevertheless, we were able to detect few antimicrobial peptides derived from milk proteins upon *S. agalactiae* or *Prototheca* spp. infection. Specifically, isracidin is a known antimicrobial peptide derived from the N-terminal region of α_{s1}-casein with the sequence RPKHPIKHQGLPQEVLNENLLRF (16−38). This peptide has proven to be effective against several microorganisms responsible for mastitis[59,60] and it is naturally present in milk, as reported by Dallas et al.[57] and Addis et al.[27] We identified an almost complete form of isracidin in positive samples (residues 16−37, RPKHPIKHQGLPQEVLNENLLR) together with two other smaller forms: RPKHPIKHQGLPQEVLNENLL (16−36) and RPKHPIKHQ (16−24). The β-casein-derived peptides casecidin-15 YQEPVLGPVRGPFPI (208−222) and casecidin-17 YQEPVLGPVRGPFPIIV (208−224) were shown to have antibacterial activity against *E. coli*[61] and they were found in their full sequences also in our study. Casecidin-15 was found only in one of the two pools of *Prototheca* spp. infected milk; therefore, it was not included in the panel of peptides potentially characteristic of the P+ category. In contrast, casecidin-17 was more abundant in positive samples ($\log_2$ FC = 3.3) and, although without the statistical significance support, especially in the P+ group samples. A similar casecidin-17 peptide lacking the C-terminal residue was also found to be significantly increased in the positive group ($\log_2$ FC = 4.7), probably as a result of the exopeptidase activity.

In conclusion, we observed that in our cohort of animals, *S. agalactiae* and *Prototheca* spp. do not show pathogen-specific milk peptidome fingerprints. Both mastitis-causing agents appear to trigger an increased proteolysis, resulting in a large number of endogenous peptides released in the milk. This could be the result of the predominant activity of host proteolytic enzymes, which may mask the effect of exogenous proteases. By applying and combining different MS-based approaches, we identified a panel of milk peptides, mainly derived from caseins, discriminant of subclinical IMI, which can be simultaneously and rapidly monitored. Upon validation on an independent and larger cohort of animals, these findings pave the way for the possible development of diagnostic tools to be applied at the herd level for the detection of subclinical infection. Differently, in order to identify specific pathogens, further studies are needed. The possibility to rapidly discriminate the causative agent could be particularly helpful to identify infected animals and prevent the spreading of infection, especially when dealing with mastitis from *Prototheca*, due to the ineffectiveness of antibiotics against this pathogenic agent. In addition, the integration of these results with those of other *-omic* technologies (e.g., transcriptomics and metagenomics) could provide further insights into the pathogen-specific physiopathology of this disease.

https://doi.org/10.1021/acs.jafc.3c03065
J. Agric. Food Chem. 2023, 71, 16827−16839

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.3c03065.

Potential discriminant features identified by MALDI-TOF mass spectrometry analysis; results of Mann−Whitney test on positive (Sa+ plus P+) and negative samples (PDF)

Peptides identified through untargeted LC-MS/MS and complete list of optimized transitions (XLSX)

■ AUTHOR INFORMATION

Corresponding Author

Giorgio Arrigoni − *Department of Biomedical Sciences, University of Padova, Padova 35131, Italy;* orcid.org/0000-0002-4103-2733; Email: giorgio.arrigoni@unipd.it

Authors

Alice Vanzin − *Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE), University of Padova, Legnaro (PD) 35020, Italy;* orcid.org/0000-0001-8215-5265

Cinzia Franchin − *Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE), University of Padova, Legnaro (PD) 35020, Italy;* orcid.org/0000-0001-9223-117X

Ilaria Battisti − *Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE), University of Padova, Legnaro (PD) 35020, Italy;* orcid.org/0009-0000-5561-6804

Antonio Masi − *Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE), University of Padova, Legnaro (PD) 35020, Italy;* orcid.org/0000-0003-0536-5984

Andrea Squartini − *Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE), University of Padova, Legnaro (PD) 35020, Italy*

Vittoria Bisutti − *Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE), University of Padova, Legnaro (PD) 35020, Italy*

Diana Giannuzzi − *Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE), University of Padova, Legnaro (PD) 35020, Italy;* orcid.org/0000-0003-2975-0385

Luigi Gallo − *Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE), University of Padova, Legnaro (PD) 35020, Italy*

Alessio Cecchinato − *Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE), University of Padova, Legnaro (PD) 35020, Italy;* orcid.org/0000-0003-3518-720X

Sara Pegolo − *Department of Agronomy, Food, Natural Resources, Animals and Environment (DAFNAE), University of Padova, Legnaro (PD) 35020, Italy;* orcid.org/0000-0001-6390-9826

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.3c03065

Funding

This research was carried out as part of the LATSAN project funded by the Ministero delle politiche agricole alimentari, forestali e del turismo (MIPAAF), Rome, Italy, and by the DOR2284954/22 granted by the University of Padova (Italy). Moreover, the study was carried out within the Agritech National Research Center and received funding from the European Union Next-GenerationEU (PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR) − MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4—D.D. 1032 17/06/2022, CN00000022). This manuscript reflects only the authors' views and opinions, neither the European Union nor the European Commission can be considered responsible for them.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank Dr. Antonio Barberio of the IZSVe for the bacteriological analyses; Prof. Matteo Gianesella of the Department of Animal Medicine, Production and Health (MAPS), University of Padova, for the clinical evaluation and veterinary consulting; Sofia Ton of the Breeders Association of the Veneto Region (ARAV, Padova, Italy) for the analyses of milk composition; the chemical laboratory La.Chi of the DAFNAE department, University of Padova, for the provision of the UHPLC—Triple quadrupole instrumentation. Alessandro Toscano, Martina Piazza, Rossella Tessari, Selene Massaro and Giorgia Secchi for the sampling activities.

■ ABBREVIATIONS

ARAV: Milk Quality Laboratory of the Breeders Association of the Veneto Region
BTN1A1: butyrophilin subfamily 1 member A1
CV: coefficient of variation
DIM: days in milk
DSCC: differential somatic cell count
FASP: filter-aided sample preparation
FC: fold change
FDR: false discovery rate
GLYCAM1: glycosylation-dependent cell adhesion molecule 1
GP2: glycoprotein 2
HCCA: α-cyano-4-hydroxycinnamic acid
IMI: intramammary infections
IZSVe: State Veterinary Laboratory for Northeastern Italy
LC-MS/MS: liquid chromatography coupled with tandem mass spectrometry
LGB: β-lactoglobulin
LPO: lactoperoxidase
MALDI-TOF: matrix-assisted laser desorption/ionization time-of-flight
MS: mass spectrometry
nano-HPLC: nano high-performance liquid chromatography
Neg: negative
P+: positive for *Prototheca* spp.
PCA: principal component analysis
Pos: positive
PLS-DA: partial last square discriminant analyses
QC: quality control
S. agalactiae: *Streptococcus agalactiae*
Sa+: positive for *S. agalactiae*
SCC: somatic cell count
SCS: somatic cell score
SNR: signal-to-noise ratio
SRM: selected reaction monitoring

T0: time zero
T1: time one
TFA: trifluoroacetic acid
TIC: total ion current
UHPLC: ultra high-performance liquid chromatography
VIP: variable importance in the projection
α_{s1}-CN: αs1-casein
αs2-CN: αs2-casein
β-CN: β-casein
κ-CN: κ-casein.

REFERENCES

(1) Maréchal, C. Le; Thiéry, R.; Vautor, E.; Loir Le, Y. Mastitis Impact on Technological Properties of Milk and Quality of Milk Products-A Review. *Dairy Sci. Technol.* **2011**, *91* (3), 247−282.

(2) Pegolo, S.; Giannuzzi, D.; Bisutti, V.; Tessari, R.; Gelain, M. E.; Gallo, L.; Schiavon, S.; Tagliapietra, F.; Trevisi, E.; Ajmone Marsan, P.; Bittante, G.; Cecchinato, A. Associations between Differential Somatic Cell Count and Milk Yield, Quality, and Technological Characteristics in Holstein Cows. *J. Dairy Sci.* **2021**, *104* (4), 4822−4836.

(3) Halasa, T.; Huijps, K.; Østerås, O.; Hogeveen, H. Economic Effects of Bovine Mastitis and Mastitis Management: A Review. *Vet. Q.* **2007**, *29* (1), 18−31.

(4) Ruegg, P. L. What Is Success? A Narrative Review of Research Evaluating Outcomes of Antibiotics Used for Treatment of Clinical Mastitis. *Front. Vet. Sci.* **2021**, *8*, 1−14.

(5) Schwarz, D.; Kleinhans, S.; Reimann, G.; Stückler, P.; Reith, F.; Ilves, K.; Pedastsaar, K.; Yan, L.; Zhang, Z.; Valdivieso, M.; Barreal, M. L.; Fouz, R. Investigation of Dairy Cow Performance in Different Udder Health Groups Defined Based on a Combination of Somatic Cell Count and Differential Somatic Cell Count. *Prev. Vet. Med.* **2020**, *183*, No. 105123.

(6) Toyotome, T.; Matsui, S. Analysis of *Prototheca* and Yeast Species Isolated from Bulk Tank Milk Collected in Tokachi District Japan. *J. Dairy Sci.* **2022**, *105* (10), 8364−8370.

(7) Jagielski, T.; Roeske, K.; Bakuła, Z.; Piech, T.; Wlazło, Ł.; Bochniarz, M.; Woch, P.; Krukowski, H. A Survey on the Incidence of *Prototheca* Mastitis in Dairy Herds in Lublin Province Poland. *J. Dairy Sci.* **2019**, *102* (1), 619−628.

(8) Zecconi, A.; dell'Orco, F.; Rizzi, N.; Vairani, D.; Cipolla, M.; Pozzi, P.; Zanini, L. Cross-Sectional Study on the Prevalence of Contagious Pathogens in Bulk Tank Milk and Their Effects on Somatic Cell Counts and Milk Yield. *Ital. J. Anim. Sci.* **2020**, *19* (1), 66−74.

(9) Shave, C. D.; Millyard, L.; May, R. C. Now for Something Completely Different: *Prototheca* Pathogenic Algae. *PLoS Pathog.* **2021**, *17* (4), 1−7.

(10) Bobbo, T.; Ruegg, P. L.; Stocco, G.; Fiore, E.; Gianesella, M.; Morgante, M.; Pasotto, D.; Bittante, G.; Cecchinato, A. Associations between Pathogen-Specific Cases of Subclinical Mastitis and Milk Yield, Quality, Protein Composition, and Cheese-Making Traits in Dairy Cows. *J. Dairy Sci.* **2017**, *100* (6), 4868−4883.

(11) Akers, R. M.; Nickerson, S. C. Mastitis and Its Impact on Structure and Function in the Ruminant Mammary Gland. *J. Mammary Gland Biol. Neoplasia* **2011**, *16* (4), 275−289.

(12) Oviedo-Boyso, J.; Valdez-Alarcón, J. J.; Cajero-Juárez, M.; Ochoa-Zarzosa, A.; López-Meza, J. E.; Bravo-Patiáo, A.; Baizabal-Aguirre, V. M. Innate Immune Response of Bovine Mammary Gland to Pathogenic Bacteria Responsible for Mastitis. *J. Infect.* **2007**, *54* (4), 399−409.

(13) Ezzat Alnakip, M.; Quintela-Baluja, M.; Böhme, K.; Fernández-No, I.; Caamaño-Antelo, S.; Calo-Mata, P.; Barros-Velázquez, J. The Immunology of Mammary Gland of Dairy Ruminants between Healthy and Inflammatory Conditions. *J. Vet. Med.* **2014**, *2014*, 1−31.

(14) Caggiano, N.; Lorenzo Smirnoff, A.; Bottini, J. M.; De Simone, E. A. Protease Activity and Protein Profile in Milk from Healthy Dairy Cows and Cows with Different Types of Mastitis. *Int. Dairy J.* **2019**, *89*, 1−5.

(15) Guerrero, A.; Dallas, D. C.; Contreras, S.; Bhandari, A.; Cánovas, A.; Islas-Trejo, A.; Medrano, J. F.; Parker, E. A.; Wang, M.; Hettinga, K.; Chee, S.; German, J. B.; Barile, D.; Lebrilla, C. B. Peptidomic Analysis of Healthy and Subclinically Mastitic Bovine Milk. *Int. Dairy J.* **2015**, *46*, 46−52.

(16) Hurley, M. J.; Larsen, L. B.; Kelly, A. L.; McSweeney, P. L. H. The Milk Acid Proteinase Cathepsin D: A Review. *Int. Dairy J.* **2000**, *10* (10), 673−681.

(17) Kelly, A. L.; O'Flaherty, F.; Fox, P. F. Indigenous Proteolytic Enzymes in Milk: A Brief Overview of the Present State of Knowledge. *Int. Dairy J.* **2006**, *16* (6), 563−572.

(18) Prin-Mathieu, C.; Le Roux, Y.; Faure, G. C.; Laurent, F.; Béné, M. C.; Moussaoui, F. Enzymatic Activities of Bovine Peripheral Blood Leukocytes and Milk Polymorphonuclear Neutrophils during Intramammary Inflammation Caused by Lipopolysaccharide. *Clin. Diagn. Lab. Immunol.* **2002**, *9* (4), 812−817.

(19) Zhao, X.; Lacasse, P. Mammary Tissue Damage during Bovine Mastitis: Causes and Control. *J. Anim. Sci.* **2008**, *86* (13 Suppl), 57−65.

(20) Fleminger, G.; Heftsi, R.; Uzi, M.; Nissim, S.; Gabriel, L. Chemical and Structural Characterization of Bacterially-Derived Casein Peptides That Impair Milk Clotting. *Int. Dairy J.* **2011**, *21* (12), 914−920.

(21) Mansor, R.; Mullen, W.; Albalat, A.; Zerefos, P.; Mischak, H.; Barrett, D. C.; Biggs, A.; Eckersall, P. D. A Peptidomic Approach to Biomarker Discovery for Bovine Mastitis. *J. Proteomics* **2013**, *85*, 89−98.

(22) Wedholm, A.; Møller, H. S.; Lindmark-Månsson, H.; Rasmussen, M. D.; Andrén, A.; Larsen, L. B. Identification of Peptides in Milk as a Result of Proteolysis at Different Levels of Somatic Cell Counts Using LC MALDI MS/MS Detection. *J. Dairy Res.* **2008**, *75* (1), 76−83.

(23) Hogarth, C. J.; Fitzpatrick, J. L.; Nolan, A. M.; Young, F. J.; Pitt, A.; Eckersall, P. D. Differential Protein Composition of Bovine Whey: A Comparison of Whey from Healthy Animals and from Those with Clinical Mastitis. *Proteomics* **2004**, *4* (7), 2094−2100.

(24) Bathla, S.; Sindhu, A.; Kumar, S.; Dubey, S. K.; Pattnaik, S.; Rawat, P.; Chopra, A.; Dang, A.; Kaushik, J. K.; Mohanty, A. K. Tandem Mass Tag (TMT)-Based Quantitative Proteomics Reveals Potential Targets Associated with Onset of Sub-Clinical Mastitis in Cows. *Sci. Rep.* **2020**, *10* (1), 1−10.

(25) Maity, S.; Das, D.; Ambatipudi, K. Quantitative Alterations in Bovine Milk Proteome from Healthy, Subclinical and Clinical Mastitis during *S. aureus* Infection. *J. Proteomics* **2020**, *223*, No. 103815.

(26) Pongthaisong, P.; Katawatin, S.; Thamrongyoswittayakul, C.; Roytrakul, S. Milk Protein Profiles in Response to *Streptococcus agalactiae* Subclinical Mastitis in Dairy Cows. *Anim. Sci. J.* **2016**, *87* (1), 92−98.

(27) Addis, M. F.; Maffioli, E. M.; Ceciliani, F.; Tedeschi, G.; Zamarian, V.; Tangorra, F.; Albertini, M.; Piccinini, R.; Bronzo, V. Influence of Subclinical Mastitis and Intramammary Infection by Coagulase-Negative Staphylococci on the Cow Milk Peptidome. *J. Proteomics* **2020**, *226*, No. 103885.

(28) Ibeagha-Awemu, E. M.; Ibeagha, A. E.; Messier, S.; Zhao, X. Proteomics, Genomics, and Pathway Analyses of *Escherichia coli* and *Staphylococcus aureus* Infected Milk Whey Reveal Molecular Pathways and Networks Involved in Mastitis. *J. Proteome Res.* **2010**, *9* (9), 4604−4619.

(29) Kusebauch, U.; Hernández-Castellano, L. E.; Bislev, S. L.; Moritz, R. L.; Røntved, C. M.; Bendixen, E. Selected Reaction Monitoring Mass Spectrometry of Mastitis Milk Reveals Pathogen-Specific Regulation of Bovine Host Response Proteins. *J. Dairy Sci.* **2018**, *101* (7), 6532−6541.

(30) Pegolo, S.; Toscano, A.; Bisutti, V.; Giannuzzi, D.; Vanzin, A.; Lisuzzo, A.; Bonsembiante, F.; Gelain, M. E.; Cecchinato, A. *Streptococcus agalactiae* and *Prototheca* Spp. Induce Different Mammary Gland Leukocyte Responses in Holstein Cows. *JDS Commun.* **2022**, *3* (4), 270−274.

(31) Pegolo, S.; Giannuzzi, D.; Piccioli Cappelli, F.; Cattaneo, L.; Gianesella, M.; Ruegg, P. L.; Trevisi, E.; Cecchinato, A. Blood Biochemical Changes upon Subclinical Intramammary Infection and

https://doi.org/10.1021/acs.jafc.3c03065
J. Agric. Food Chem. 2023, 71, 16827−16839

Inflammation in Holstein Cattle. *J. Dairy Sci.* **2023**, DOI: 10.3168/jds.2022-23155.
(32) Isobe, N.; Kurose, T.; Suzuki, N.; Koshiishi, T.; Ueno, K.; Hisaeda, K. Effect of Oral Administration of Colostrum on Inflammation in the Udders of Dairy Cows Suffering from Mastitis. *J. Vet. Med. Sci.* **2022**, *84* (1), 59−63.
(33) National Mastitis Council (NMC). *Laboratory Handbook on Bovine Mastitis Rev. Ed.*; National Mastitis Council Inc., 2017.
(34) Pegolo, S.; Tessari, R.; Bisutti, V.; Vanzin, A.; Giannuzzi, D.; Gianesella, M.; Lisuzzo, A.; Fiore, E.; Barberio, A.; Schiavon, E.; Trevisi, E.; Piccioli Cappelli, F.; Gallo, L.; Ruegg, P.; Negrini, R.; Cecchinato, A. Quarter-Level Analyses of the Associations among Subclinical Intramammary Infection and Milk Quality, Udder Health, and Cheesemaking Traits in Holstein Cows. *J. Dairy Sci.* **2022**, *105* (4), 3490−3507.
(35) Ali, A. K. A.; Shook, G. E. An Optimum Transformation for Somatic Cell Concentration in Milk. *J. Dairy Sci.* **1980**, *63* (3), 487−490.
(36) Wiśniewski, J. R. Filter-Aided Sample Preparation for Proteome Analysis. *Methods Mol. Biol.* **2018**, *1841*, 3−10.
(37) Gibb, S.; Strimmer, K. Maldiquant: A Versatile R Package for the Analysis of Mass Spectrometry Data. *Bioinformatics* **2012**, *28* (17), 2270−2271.
(38) Palarea-Albaladejo, J.; McLean, K.; Wright, F.; Smith, D. G. E. MALDIrppa: Quality Control and Robust Analysis for Mass Spectrometry Data. *Bioinformatics* **2018**, *34* (3), 522−523.
(39) Gibb, S.; Strimmer, K. Mass Spectrometry Analysis Using MALDIquant. *Stat. Anal. Proteomics, Metabolomics, Lipidomics Data Using Mass Spectrom.* **2017**, 101−124.
(40) *T2d/RAW Converter*. Http://Www.Scripps.Edu/New_yates/Sites/Ygao//Index.Html.
(41) Gaujoux, R.; Seoighe, C. A Flexible R Package for Nonnegative Matrix Factorization. *BMC Bioinformatics* **2010**, *11*, 11.
(42) Lê, S.; Josse, J.; Husson, F. FactoMineR: An R Package for Multivariate Analysis. *J. Stat. Softw.* **2008**, *25* (1), 1−18.
(43) Rohart, F.; Gautier, B.; Singh, A.; Lê Cao, K. A. MixOmics: An R Package for 'omics Feature Selection and Multiple Data Integration. *PLoS Comput. Biol.* **2017**, *13* (11), 1−19.
(44) Käll, L.; Canterbury, J. D.; Weston, J.; Noble, W. S.; MacCoss, M. J. Semi-Supervised Learning for Peptide Identification from Shotgun Proteomics Datasets. *Nat. Methods* **2007**, *4* (11), 923−925.
(45) Aita, A.; Battisti, I.; Contran, N.; Furlan, S.; Padoan, A.; Franchin, C.; Barbaro, F.; Cattelan, A. M.; Zambon, C. F.; Plebani, M.; Basso, D.; Arrigoni, G. Salivary Proteomic Analysis in Asymptomatic and Symptomatic SARS-CoV-2 Infection: Innate Immunity, Taste Perception and FABP5 Proteins Make the Difference. *Clin. Chim. Acta* **2022**, *537*, 26−37.
(46) Adams, K. J.; Pratt, B.; Bose, N.; Dubois, L. G.; St John-Williams, L.; Perrott, K. M.; Ky, K.; Kapahi, P.; Sharma, V.; Maccoss, M. J.; Moseley, M. A.; Colton, C. A.; Maclean, B. X.; Schilling, B.; Thompson, J. W. Skyline for Small Molecules: A Unifying Software Package for Quantitative Metabolomics. *J. Proteome Res.* **2020**, *19* (4), 1447−1458.
(47) Team, R. C. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2014. R Foundation for Statistical Computing *R Found. Stat. Comput.* 2015, *2* 2019.
(48) Thomas, F. C.; Mullen, W.; Tassi, R.; Ramı, A.; Mudaliar, M.; Mcneilly, T. N.; Zadoks, R. N.; Burchmore, R.; Eckersall, P. D. Mastitomics, the Integrated Omics of Bovine Milk in an Experimental Model of *Streptococcus uberis* Mastitis: 1. High Abundance Proteins, Acute Phase Proteins and Peptidomics. *Mol. Biosyst.* **2016**, *12*, 2735−2747.
(49) Leitner, G.; Krifucks, O.; Merin, U.; Lavi, Y.; Silanikove, N. Interactions between Bacteria Type, Proteolysis of Casein and Physico-Chemical Properties of Bovine Milk. *Int. Dairy J.* **2006**, *16* (6), 648−654.
(50) Le Roux, Y.; Laurent, F.; Moussaoui, F. Polymorphonuclear Proteolytic Activity and Milk Composition Change. *Vet. Res.* **2003**, *34* (5), 629−645.
(51) Napoli, A.; Aiello, D.; Di Donna, L.; Prendushi, H.; Sindona, G. Exploitation of Endogenous Protease Activity in Raw Mastitic Milk by MALDI-TOF/TOF. *Anal. Chem.* **2007**, *79* (15), 5941−5948.
(52) Dallas, D. C.; Guerrero, A.; Parker, E. A.; Garay, L. A.; Bhandari, A.; Lebrilla, C. B.; Barile, D.; German, J. B. Peptidomic Profile of Milk of Holstein Cows at Peak Lactation. *J. Agric. Food Chem.* **2014**, *62* (1), 58−65.
(53) Åkerstedt, M.; Wredle, E.; Lam, V.; Johansson, M. Protein Degradation in Bovine Milk Caused by *Streptococcus agalactiae*. *J. Dairy Res.* **2012**, *79* (3), 297−303.
(54) Caggiano, N.; Smirnoff, A. L.; Bottini, J. M.; Simone De, E. A. Protease Activity and Protein pro Fi Le in Milk from Healthy Dairy Cows and Cows with Different Types of Mastitis. *Int. Dairy J.* **2019**, *89*, 1−5.
(55) Bisutti, V.; Vanzin, A.; Toscano, A.; Pegolo, S.; Giannuzzi, D.; Gallo, L.; Trevisi, E.; Negrini, R.; Cecchinato, A.; Tagliapietra, F.; Schiavon, S. Impact of Somatic Cell Count Combined with Differential Somatic Cell Count on Milk Protein Fractions in Holstein Cattle. *J. Dairy Sci.* **2022**, *105* (8), 6447−6459.
(56) Boehmer, J. L.; Bannerman, D. D.; Shefcheck, K.; Ward, J. L. Proteomic Analysis of Differentially Expressed Proteins in Bovine Milk during Experimentally Induced *Escherichia coli* Mastitis. *J. Dairy Sci.* **2008**, *91* (11), 4206−4218.
(57) Dallas, D. C.; Guerrero, A.; Khaldi, N.; Castillo, P. A.; Martin, W. F.; Smilowitz, J. T.; Bevins, C. L.; Barile, D.; German, J. B.; Lebrilla, C. B. Extensive in Vivo Human Milk Peptidomics Reveals Specific Proteolysis Yielding Protective Antimicrobial Peptides. *J. Proteome Res.* **2013**, *12* (5), 2295−2304.
(58) Addis, M. F.; Bronzo, V.; Puggioni, G. M. G.; Cacciotto, C.; Tedde, V.; Pagnozzi, D.; Locatelli, C.; Casula, A.; Curone, G.; Uzzau, S.; Moroni, P. Relationship between Milk Cathelicidin Abundance and Microbiologic Culture in Clinical Mastitis. *J. Dairy Sci.* **2017**, *100* (4), 2944−2953.
(59) Hayes, M.; Ross, R. P.; Fitzgerald, G. F.; Hill, C.; Stanton, C. Casein-Derived Antimicrobial Peptides Generated by *Lactobacillus acidophilus* DPC6026. *Appl. Environ. Microbiol.* **2006**, *72* (3), 2260−2264.
(60) Mohanty, D.; Jena, R.; Choudhury, P. K.; Pattnaik, R.; Mohapatra, S.; Saini, M. R. Milk Derived Antimicrobial Bioactive Peptides: A Review. *Int. J. Food Prop.* **2016**, *19* (4), 837−846.
(61) Birkemo, G. A.; O'Sullivan, O.; Ross, R. P.; Hill, C. Antimicrobial Activity of Two Peptides Casecidin 15 and 17, Found Naturally in Bovine Colostrum. *J. Appl. Microbiol.* **2009**, *106* (1), 233−240.