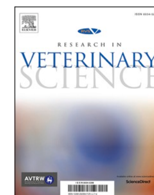
Research in Veterinary Science 140 (2021) 91–99

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc



Changes in saliva proteins in cows with mastitis: A proteomic approach

L. Franco-Martínez [a], A. Muñoz-Prieto [b], M.D. Contreras-Aguilar [a], R. Želvytė [c], I. Monkevičienė [c], A. Horvatić [d], J. Kuleš [b], V. Mrljak [b], J.J. Cerón [a], D. Escribano [a,e,*]

[a] Interdisciplinary Laboratory of Clinical Analysis, Interlab-UMU, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, 30100, Espinardo, Murcia, Spain
[b] Internal Diseases Clinic, Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, 10 000 Zagreb, Croatia
[c] Department of Anatomy and Physiology, Research Center of Digestive Physiology and Pathology, Veterinary Academy, Lithuanian University of Health Sciences, Tilzes str. 18, LT-47181 Kaunas, Lithuania
[d] Department of Chemistry and Biochemistry, Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, 10 000 Zagreb, Croatia
[e] Department of Animal Production, Veterinary School, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, 30100, Espinardo, Murcia, Spain

A R T I C L E I N F O

Keywords:
Biomarkers
Bovine
Mastitis
Saliva
Serum
Tandem Mass Tags

A B S T R A C T

This study aimed to evaluate the possible saliva proteome changes in cows with mastitis using a Tandem Mass Tags (TMT) proteomics approach. For this purpose, the salivary proteomes from healthy cows and cows with mastitis were analysed, and their serum proteomes were also studied for comparative purposes. A total of eight saliva and serum paired samples for each group were used for the proteomic study, and eight additional samples for each group were analysed in the analytical and overlap performance studies. In saliva samples, 2192 proteins were identified, being sixty-three differentially modulated in mastitis. In serum, 1299 proteins were identified, being twenty-nine differentially modulated in mastitis. Gamma glutamyl transferase (γGT) in saliva and serum amyloid A (SAA) were validated by commercially available automated assays.

In conclusion, there are changes in protein expression and metabolic pathways in saliva and serum proteomes of cows with mastitis, showing different response patterns but complementary information.

1. Introduction

Mastitis is the most frequent infectious disease worldwide in dairy cows and a major problem in the global dairy industry by reducing animals' welfare, producing high costs to farmers, and being the primary cause of the use of antibiotics in dairy herds (Halasa et al., 2007; Thomas et al., 2016).

Proteomic techniques allow the identification of a large number of proteins present in a tissue or biofluid, having a high number of potential applications in veterinary sciences, both in clinics and in animal production (Almeida et al., 2015). The high-resolution quantitative gel-free proteomics technology that uses isobaric tagging options such as Tandem Mass Tags (TMT) is highly sensitive and reproducible, allowing the simultaneous quantification of thousands of labelled proteins (Baeumlisberger et al., 2010; Dayon et al., 2011; Giron et al., 2011). This technique has been recently applied in dogs, pigs, and sheep, allowing the identification of new salivary biomarkers in infectious diseases, inflammatory conditions, and stress (Escribano et al., 2019a, 2019b; Franco-Martínez et al., 2020a).

Saliva is a fluid gaining interest in the last years since it can be collected by non-invasive, inexpensive and easy sampling methods, allowing the collection of multiple/repeated specimens at anytime, anywhere, and without the need for specialized staff (Contreras-Aguilar et al., 2019a, 2019b). On the other hand, unlike of milk samples, it does not need filtrations or long and intensive centrifugation to remove the high lipid content. In this line, the measurement of a profile of analytes in saliva ("sialochemistry") can be included in the same form that a biochemistry profile performed in serum/plasma (Contreras-Aguilar et al., 2020). This fact allows exploring a relatively high number of analytes that could be of interest to evaluate this frequent disease in cows. In addition, it provides information about the health status not only in cows but also in others species of animals (Lamy and Mau, 2012). There is a report in which the salivary proteomes in healthy cows have been studied (Ang et al., 2011). Also, it has been suggested that the

* Corresponding author at: Interdisciplinary Laboratory of Clinical Analysis, Interlab-UMU, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, 30100, Espinardo, Murcia, Spain.
E-mail address: det20165@um.es (D. Escribano).

https://doi.org/10.1016/j.rvsc.2021.08.008
Received 1 March 2021; Received in revised form 5 July 2021; Accepted 6 August 2021
Available online 10 August 2021
0034-5288/© 2021 Elsevier Ltd. All rights reserved.

expression of proteins in this fluid in cows could be useful for the detection of infectious status of diseases such as paratuberculosis (Mallikarjunappa et al., 2019). Besides, a recent study performed by Contreras-Aguilar et al. (2019b) showed changes in a number of salivary analytes in cows with mastitis using spectrophotometric assays. However, despite the importance of this disease and the potential applications of salivary proteomics, to the authors' knowledge, no studies in saliva proteome have been made in cows with mastitis.

The hypothesis of our study was that the application of a high-resolution quantitative proteomic technique could allow the identification of new pathways and proteins differentially represented in saliva of cows with mastitis. Therefore, our objective was to study the proteome's possible changes in the saliva of cows with mastitis using TMT proteomics. For this purpose, the proteome of saliva from healthy cows and cows with mastitis was analysed. Additionally, the proteome of serum was studied for comparative purposes. It is expected that this study will contribute to gain a more detailed understanding of the physiopathological changes associated with mastitis in cows and to elucidate the potential changes in saliva proteome due to this disease.

2. Materials and methods

2.1. Animal and sampling procedures

This study was performed using aliquots of saliva and serum samples from cows that have been described in a previous report by Contreras-Aguilar et al. (2019b). The cows were from a dairy farm in Panevezys, Lithuania, where the housing system is DeLaval (DeLaval, Sweden) based on free-stall.

Animals were divided into two groups according to their clinical condition: healthy cows (control group $n = 16$) without any clinical signs neither abnormalities in milk analysis (somatic cell count SCC < 150,000 cells/ml; Vissio et al., 2014); and cows with clinical mastitis that was recognised by observation and palpation of the udder and with presence of clots and pathogens such as *Streptococcus uberis* and/or *Steptococcus agalacitiae* in milk (mastitis group n = 16). The animals did not present any clinical evidence of acute or chronic disease other than mastitis at physical examination, they did not have fever, and the cows were not treated with any medicine prior to sampling. After the study, the animals received a standard treatment and all the animals used had a good prognosis and recovered from the infection. Of those, eight paired saliva and serum samples from each group were randomly selected for the proteomic study. The remaining eight samples from each group were used for the analytical and overlap validation studies.

The saliva samples were collected before the blood samples to avoid the possible stress induction in all cases. In the case of animals with mastitis, samples were collected within three hours of mastitis being detected. Saliva samples were obtained by placing a sponge clipped to a flexible thin metal rod into the cows' mouth for 1 min, collecting around of 1 mL of saliva. Then, the sponge was placed in plastic tubes (Salivette; Sarstedt, Aktiengesellschaft & Co., Nümbrecht, Germany) and centrifuged as previously reported (Giese et al., 2018; Contreras-Aguilar et al., 2019a, 2019b). Blood samples were collected from the jugular vein into tubes, without any additives (BD Vacutainer, Dickinson & Co., USA), let to clot at room temperature for 30 min, and centrifuged at 2000 ×*g* for 10 min. The saliva and serum supernatant was aliquoted and kept at −80 °C until proteomic analysis (Internal Disease Clinic, Faculty of Veterinary Medicine, University of Zagreb. Zagreb, Croatia).

2.2. Proteomic analysis and Liquid Chromatography-Mass Spectrometry (LC–MS/MS)

For the proteomic study, eight paired saliva and serum samples from each group were acetone-precipitated (six volumes of ice-cold acetone VWR Crop., Radnor, PA, USA) overnight, dissolved in 100 mM triethylammonium bicarbonate (TEAB) (pH 8.5) and protein concentration was obtained by colorimetric Bradford assay. A pooled sample, generated by mixing equal protein quantity of all samples for saliva or serum were employed as an internal standard for each TMT six-plex experiments.

For each sample, proteins were submitted to reduction, alkylation, digestion, and labelled using TMT six-plex reagents following manufacturer instructions (Thermo Fisher Scientific, Waltham, MA, USA) with some modifications, as reported by Martinez-Subiela et al. (2017). In short, 35 μg of proteins were reduced with 200 mM 1,4-Dithiothreitol (DTT) (Sigma-Aldrich, S t. Louis, MO, USA), alkylated with 375 mM iodoacetamide (Sigma-Aldrich, S t. Louis, MO, USA) and precipitated with ice-cold acetone overnight. Saliva and serum samples were then centrifuged and the acetone was eliminated by decantation. Afterward, 50 μL of 100 mM TEAB buffer were used to resuspend the pellets, and they were digested with trypsin (Promega Corp., Madison, WI, USA) overnight at 37 °C (trypsin-to-protein ratio 1:35, *w*/w). The reagents for TMT labelling were equilibrated and resuspended with anhydrous acetonitrile LC–MS grade (Thermo Fisher Scientific, Waltham, MA, USA) and added to each saliva and serum sample. The labelling reaction was incubated for one hour (at room temperature) and then 5% hydroxylamine (Thermo Fisher Scientific, Waltham, MA, USA) was added to each sample for its quenching. Samples were mixed at same amounts (5 μg), vacuum-dried and kept at −80 °C before subsequent LC–MS/MS analysis.

The LC − MS/MS analysis was performed using the Dionex Ultimate 3000 RSLC nano flow system (Dionex, Camberley, UK) and the Orbitrap Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), as reported by Horvatić et al. (2018). For protein identification and relative quantification, Proteome Discoverer (version 2.0., Thermo Fisher Scientific, Waltham, MA, USA) was used for the SEQUEST search against *B. taurus* FASTA files downloaded from the NCBI database (20/05/2019, 70,016 sequences) according to parameters set as follows: precursor and fragment mass tolerances of 10 ppm and 0.02 Da, two trypsin missed cleavage sites, respectively; carbamidomethyl (C), oxidation (M), fixed peptide modification, deamidation (N, Q) and TMT six-plex (K, peptide N-terminus) dynamic modifications. The false discovery rate (FDR) for peptide identification was calculated using the Percolator algorithm. Proteins with at least two unique peptides and 5% FDR were considered successfully identified. Protein quantification was based on relative intensities of reporter ions representing differentially labelled peptides selected for MS/MS fragmentation. To compare the relative quantification data between the TMT six-plex experiments, an internal standard was used.

2.3. Validation of γ-Glutamyl Transferase (γGT) in saliva and Serum Amyloid A (SAA) in serum

Based on the proteomic results, one protein in saliva (γGT) and one in serum (SAA) were selected for the validation.

The γGT in saliva was analysed using a commercial kit from Beckman (OSR6119 Beckman Coulter Inc., Fullerton, CA, USA) and was carried out by an automatic biochemical analyser (Olympus UA600, Olympus Diagnostica GmbH, Freiburg, Germany).

The SAA in serum was measured using a commercial method (VET-SAA; EIKEN Chemical Co., LTD; Tokyo, Japan) according to manufacturer instructions. This is an optical measurement method based on the latex agglutination reaction, and is measured with an automatic analyser for biochemical assay (Olympus UA600, Olympus Diagnostica GmbH, Freiburg, Germany).

The analytical validation of both methods was based on evaluating:

Precision. (1) The intra-assay coefficient of variation (CV) was calculated after analysing two pools of different concentrations (one pool of four samples with low concentrations and one pool of four samples with high concentrations) five times in a single assay-run. (2) Inter-assay CV was determined by analysing the same samples in five separate runs carried out on five consecutive days. (3) Accuracy of the assay was indirectly evaluated by linearity under dilution. Thus, two

serum and saliva samples from two healthy cows with high concentrations were serially diluted with saline solution. (4) The limit of detection (LD) was defined as the lowest analyte concentration that could be distinguished from a specimen of zero value. It was calculated based on data from 10 replicate determinations of the zero standard (saline solution) as mean value plus three standard deviations (SD).

In addition to the analytical validation, an overlap performance study was made to test the assays' ability to give different values using eight cows with mastitis compared to eight healthy cows.

2.4. Statistical and Gene Ontology (GO) analyses of proteomic data

All statistics were performed using R v3.2.2 (RC, 2013). First, proteins with less than two unique peptides and the ones with 100% missing data were removed from the analysis. Sample outliers were detected per each group for each protein using the Dixon's test from R package outlier's v0.14.[1] If any sample outlier was significant ($p < 0.05$) it was removed from further analysis. As most of the analysed proteins did not follow a normal distribution, tested by the Shapiro-Wilk test, to test the difference in protein abundance between groups, the Wilcoxon-Mann-Whitney test was performed. Fold change (FC) between the two groups was calculated as mean (mastitis)/mean (healthy) and expressed on log2 scale.

Proteins GI accession numbers were converted into official gene symbol either by DAVID conversion tool (https://david.ncifcrf.gov/conversion.jsp), UniProtKB ID mapping (https://www.uniprot.org/uploadlists/) or from Mascot search engine implemented into Proteome Discoverer.

Genes encoding the differentially expressed proteins between mastitis and healthy control groups were used to determine the GO terms over-represented in mastitis using Protein Analysis Through Evolutionary Relationships (PANTHER) classification tool (http://www.pantherdb.org/).

2.5. Statistical analysis for γGT and SAA validation

The statistical analysis was performed using routine descriptive statistical procedures and software (Graph Pad Prism, Version 9; SPSS15.0, SPSS Inc., Chicago, IL, USA). The CV (%) of the assay was calculated as SD divided by the mean value of analysed replicates and multiplied by 100. Linear regression analyses were performed to assess the linearity under dilution. SAA and *γGT* results of the overlap performance study were evaluated for approximate normality of distribution using the Shapiro-Wilk normality test statistics. As data were no normally distributed, differences in SAA and *γGT* concentration between healthy and cows with mastitis were evaluated using unpaired and non-parametric Wilcoxon-Mann-Whitney test for the overlap performance study. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Proteomic analysis

3.1.1. Saliva

A total of 2192 proteins were identified in saliva. Sixty-three proteins showed significant differences between the groups of animals (Table 1). Forty-five of them were up-regulated proteins in cows with mastitis, being the major proteins that appear (classified in Table 1 by the magnitude of change in the order of the higher to the lower FC): Lung and nasal epithelium carcinoma-associated protein (BSP30b, FC = 1.561, and $p = 0.004$), keratin (KRT, FC = 1.212, and $p = 0.021$), lysozyme (LYZ, FC = 1.172, and $p = 0.032$), beta-lactoglobulin (Beta-LG,

[1] See: Komsta, L. Package 'outliers'. R Top. Doc. 2015. https://cran.r-project.org/web/packages/outliers/ (accessed on 15 January 2021).

Table 1

Statistically significant expression changes of proteins in saliva between a group of healthy cows ($n = 8$) in relation to a mastitis group (n = 8). Ordained based on fold change between groups.

Accession ID	Protein name	*p* value	Mean (SD) healthy	Mean (SD) mastitis	log2 (fold change)
296,481,016	Short palate, lung and nasal epithelium carcinoma-associated protein 2B precursor	0.004	0.25 (0.048)	0.738 (0.705)	1.561
134,024,768	Keratin 4 protein	0.021	0.642 (0.089)	1.488 (1.154)	1.212
1,423,516,689	Chain A, Short palate, lung and nasal epithelium carcinoma-associated protein 2B	0.007	0.353 (0.074)	0.797 (0.58)	1.174
1,516,608,593	Chain B, Short palate, lung and nasal epithelium carcinoma-associated protein 2B	0.007	0.353 (0.074)	0.797 (0.58)	1.174
296,487,701	lysozyme 14D	0.032	0.736 (0.172)	1.659 (0.525)	1.172
741,926,420	Lysozyme C, tracheal isozyme isoform X1	0.032	0.736 (0.172)	1.659 (0.525)	1.172
1,387,270,172	Lysozyme C, tracheal isozyme isoform X2	0.032	0.736 (0.172)	1.659 (0.525)	1.172
74,268,307	Intestinal lysozyme	0.032	0.736 (0.172)	1.659 (0.525)	1.172
118,151,378	Lysozyme C, milk isozyme precursor	0.032	0.736 (0.172)	1.659 (0.525)	1.172
674,274,855	Lysozyme F1	0.032	0.736 (0.172)	1.659 (0.525)	1.172
674,274,859	Lysozyme F3	0.032	0.736 (0.172)	1.659 (0.525)	1.172
741,963,799	Keratin, type I cytoskeletal 24 isoform X1	0.014	0.787 (0.103)	1.688 (1.019)	1.100
296,476,400	Keratin 27	0.014	0.787 (0.103)	1.688 (1.019)	1.100
122,692,323	Lysozyme C, tracheal isozyme precursor	0.003	0.655 (0.15)	1.385 (0.501)	1.080
1,374,502,767	Chain A, Beta-lactoglobulin	0.040	0.651 (0.052)	1.364 (0.666)	1.067
520	Beta-lactoglobulin	0.040	0.65 (0.051)	1.359 (0.664)	1.064
162,748	Beta-lactoglobulin, partial	0.040	0.65 (0.051)	1.359 (0.664)	1.064
49,259,423	Chain X, Beta-lactoglobulin	0.040	0.65 (0.051)	1.359 (0.664)	1.064
195,957,138	Major allergen beta-lactoglobulin	0.040	0.65 (0.051)	1.359 (0.664)	1.064
296,482,162	Beta-lactoglobulin precursor	0.040	0.65 (0.051)	1.359 (0.664)	1.064
347,447,468	Chain B, Beta-lactoglobulin	0.040	0.65 (0.051)	1.359 (0.664)	1.064
1,387,197,304	Beta-lactoglobulin isoform X1	0.040	0.65 (0.051)	1.359 (0.664)	1.064
1,391,852,346	Chain D, Major allergen beta-lactoglobulin	0.040	0.65 (0.051)	1.359 (0.664)	1.064
1,562,366,904	Chain C, Beta-lactoglobulin	0.040	0.65 (0.051)	1.359 (0.664)	1.064
296,487,872	Keratin 6A	0.015	0.773 (0.175)	1.44 (0.719)	0.897
1,393,170,126	Keratin, type I cytoskeletal 14	0.028	0.75 (0.153)	1.395 (0.609)	0.895
162,797	Beta-casein precursor	0.015	0.75 (0.144)	1.353 (0.786)	0.851
162,805	Beta-casein	0.015	0.75 (0.144)	1.353 (0.786)	0.851
459,292	Beta-casein A3	0.015			0.851

(continued on next page)

Table 1 (*continued*)

Accession ID	Protein name	*p* value	Mean (SD) healthy	Mean (SD) mastitis	log2 (fold change)
			0.75 (0.144)	1.353 (0.786)	
1,387,273,717	Beta-casein isoform X1	0.015	0.75 (0.144)	1.353 (0.786)	0.851
1,387,273,719	Beta-casein isoform X2	0.015	0.75 (0.144)	1.353 (0.786)	0.851
134,140	S100 calcium-binding protein A2	0.006	0.735 (0.154)	1.276 (0.463)	0.795
1,387,262,348	Small proline-rich protein 2E	0.001	0.791 (0.216)	1.313 (0.297)	0.731
1,537,751,055	S100 calcium binding protein A12	0.005	0.807 (0.196)	1.299 (0.37)	0.686
528,942,110	Protein S100-A12 isoform X1	0.007	0.803 (0.213)	1.283 (0.384)	0.676
479	Cytokeratin VIb	0.015	0.879 (0.12)	1.374 (0.595)	0.644
151,553,925	Keratin 10 (epidermolytic hyperkeratosis; keratosis palmaris et plantaris)	0.015	0.879 (0.12)	1.374 (0.595)	0.644
296,476,308	Keratin, type I cytoskeletal 10	0.015	0.879 (0.12)	1.374 (0.595)	0.644
296,481,047	Protein-glutamine gamma-glutamyltransferase E	0.038	0.852 (0.283)	1.228 (0.394)	0.527
1,387,203,516	Protein-glutamine gamma-glutamyltransferase E isoform X1	0.038	0.852 (0.283)	1.228 (0.394)	0.527
296,487,899	Keratin, type II cytoskeletal 5	0.015	0.837 (0.16)	1.199 (0.325)	0.518
27,806,501	Protein disulfide-isomerase precursor	0.050	0.891 (0.347)	1.211 (0.201)	0.442
148,878,430	Protein disulfide-isomerase	0.050	0.891 (0.347)	1.211 (0.201)	0.442
529,016,270	Odorant-binding protein	0.021	0.806 (0.112)	1.056 (0.258)	0.389
359,076,495	WAP four-disulfide core domain protein 18	0.038	1.046 (0.052)	1.133 (0.098)	0.115
296,491,153	Profilin 1	0.038	1.176 (0.33)	0.898 (0.153)	−0.389
296,481,967	Von Ebner gland protein	0.021	1.061 (0.27)	0.754 (0.19)	−0.492
296,473,967	Leukocyte elastase inhibitor	0.028	1.032 (0.35)	0.726 (0.148)	−0.507
146,386,603	Chain C, Actin, Cytoplasmic 1	0.021	1.137 (0.364)	0.788 (0.064)	−0.528
334,878,521	Chain A, Beta-Actin	0.021	1.137 (0.364)	0.788 (0.064)	−0.528
385,251,867	Chain A, Actin, Cytoplasmic 1	0.021	1.137 (0.364)	0.788 (0.064)	−0.528
296,473,952	Serpin peptidase inhibitor, clade B, member 1	0.011	1.205 (0.391)	0.802 (0.191)	−0.587
296,491,743	Cathelicidin	0.028	1.198 (0.369)	0.785 (0.204)	−0.609
162,731	Bactenecin 5 precursor	0.050	1.219 (0.438)	0.788 (0.237)	−0.629
183,240,946	Cathelicidin 2, partial	0.050	1.219 (0.438)	0.788 (0.237)	−0.629
46,576,685	Prolactin-inducible protein homolog	0.028	1.236 (0.436)	0.791 (0.256)	−0.643
77,736,341	Antithrombin-III precursor	0.003	1.222 (0.238)	0.781 (0.254)	−0.645
157,838,186	Chain B, Crystal Structure Of Cleaved Bovine Antithrombin Iii At 3.2 Angstroms Resolution	0.003	1.222 (0.238)	0.781 (0.254)	−0.645
148,744,287	Cathelicidin 5	0.021			−0.674

Table 1 (*continued*)

Accession ID	Protein name	*p* value	Mean (SD) healthy	Mean (SD) mastitis	log2 (fold change)
			1.178 (0.443)	0.738 (0.25)	
1,387,234,898	Cathelicidin-5 isoform X1	0.021	1.178 (0.443)	0.738 (0.25)	−0.674
208,969,128	Cathelicidin 6	0.028	1.218 (0.437)	0.741 (0.244)	−0.716
296,491,742	Cathelicidin-4 precursor	0.004	1.182 (0.316)	0.681 (0.225)	−0.795
296,480,949	Trappin-6	0.021	1.116 (0.59)	0.435 (0.228)	−1.359

FC = 1.067, and p = 0.040), beta-casein (CSN, FC = 0.851, and p = 0.015), S100 calcium-binding protein (S100A, FC = 0.795, and p = 0.006), small proline-rich protein (SPRR, FC = 0.731, and p = 0.001), protein-glutamine gamma-glutamyltransferase (γGT, FC = 0.527, and p = 0.038), protein disulfide-isomerase odorant-binding protein (OBP, FC = 0.389, and p = 0.021), and WAP four-disulfide core domain protein (WFDC, FC = 0.115, and p = 0.038). Eighteen proteins were statistically significantly down-regulated in cows with mastitis, being the major proteins that appear (classified in Table 1 by the magnitude of change in the order of the higher to the lower FC): Profilin 1 (PFN1, FC = −0.389, and p = 0.038), von Ebner gland protein (VEMSGP, FC = −0.492, and p = 0.021), leukocyte elastase inhibitor (LEI, FC = −0.507, and p = 0.028), actin (ACTN, FC = −0.528, and p = 0.021), serpin peptidase inhibitor (SERPINB9, FC = −0.587, and p = 0.011), cathelicidin (CAMP, FC = −0.609, and p = 0.028), bactenecin (Bac5, FC = −0.629, and p = 0.050), prolactin-inducible protein homolog (PIP, FC = −0.643, and p = 0.028), antithrombin-III precursor (ATIII, FC = −0.645, and p = 0.003), and trappin-6 (TRAP6, FC = −1359, and p = 0.021).

3.1.2. *Serum*

A total of 1299 proteins were identified in serum samples. Twenty-nine proteins showed significant differences ($p < 0.05$) in cows with mastitis concerning the healthy group (Table 2). Nineteen of them were up-regulated proteins in cows with mastitis, being the major proteins found (ordered from greater to the lower FC): Serum amyloid A (SAA, FC = 1.423, and p = 0.001), serpin A3–5 (SERPINA 3–5, FC = 0.848, and p = 0.030), immunoglobulin lambda (IGLL, FC = 0.595, and p = 0.004) and kappa (IGKC, FC = 0.444, and p = 0.021), alpha1-antichymotrypsin (ACT, FC = 0.363, and p = 0.021), vitamin K-dependent protein Z (PROZ, FC = 0.323, p = 0.017), complement factor (CFP, FC = 0.273, and p = 0.017) and prothrombin (F2, FC = 0.164, and p = 0.021). Other ten proteins were down-regulated in cows with mastitis, being the major proteins found (ordered from greater to the lower FC): Complement C4 (CA, FC = −0.129, and p = 0.029), pregnancy zone protein isoform X2 (PZP, FC = −0.230, and p = 0.040), phosphatidylinositol-glycan-specific phospholipase D (PI-G PLD, FC = −0256, and p = 0.016), serum albumin (ALB, FC = −0.259, and p = 0.010) and plasma retinol-binding protein (RBP4, FC = −0.445, and p = 0.009).

3.2. *Validation results of salivary γGT*

3.2.1. *Analytical validation*

In the pool with high *γGT* activity the intra-assay CV was 7.3% (mean 64 ± 5.0 SD UI/L) , whereas in the pool with low activity it was 9.2% (mean 10.2 ± 1.0 SD UI/L). The inter-assays CVs were 9.1% for the pool with high *γGT* activity (mean 58 ± 5.3 SD UI/L) and 14.7% for the pool with low activity (mean 11.8 ± 1.7 SD UI/L). In the linearity under dilution study (1:1 to 1:32), the two samples showed regression coefficients close to 1: 0.96 and 0.99. The analytical limit of detection observed was 0.8 UI/L.

Table 2
Statistically significant expression changes of proteins in serum between a group of healthy cows ($n = 8$) in relation to a mastitis group ($n = 8$). Ordained based on fold-change between groups.

Accession ID	Protein name	p value	Mean (SD) healthy	Mean (SD) mastitis	log2 (fold change)
245,183	Amyloid fibril protein A, AA [cattle, kidney, Peptide, 80 aa]	0.001	0.58 (0.273)	1.555 (0.698)	1.423
245,184	Serum amyloid A, SAA [cattle, serum, Peptide, 111 aa]	0.001	0.614 (0.263)	1.53 (0.632)	1.317
1,387,249,331	Serum amyloid A protein isoform X1	0.001	0.614 (0.263)	1.53 (0.632)	1.317
1,387,233,947	Serpin A3–5	0.030	0.765 (0.301)	1.377 (0.688)	0.848
313,471,467	Serpin A3–4	0.014	0.817 (0.234)	1.384 (0.633)	0.760
226,373,739	Serpin A3–6 precursor	0.009	0.83 (0.193)	1.381 (0.618)	0.735
1,276,599	Immunoglobulin lambda light chain variable region, partial	0.004	0.819 (0.069)	1.237 (0.329)	0.595
343,482,369	Immunoglobulin kappa light chain constant region, partial	0.021	0.855 (0.238)	1.163 (0.258)	0.444
115,545,495	IGK protein	0.021	0.861 (0.236)	1.168 (0.252)	0.440
121,531,636	Serpin A3–8	0.029	0.906 (0.155)	1.214 (0.286)	0.422
86,438,018	Serpin A3 protein	0.006	0.808 (0.133)	1.041 (0.164)	0.366
538,275	Alpha1-antichymotrypsin isoform pHHK11, partial	0.021	0.905 (0.144)	1.164 (0.276)	0.363
1,387,202,994	Vitamin K-dependent protein Z	0.017	0.926 (0.083)	1.158 (0.164)	0.323
31,340,900	Serpin A3–1 precursor	0.029	0.807 (0.131)	0.995 (0.116)	0.302
296,470,740	Complement factor properdin	0.017	1.028 (0.099)	1.242 (0.076)	0.273
27,806,947	Prothrombin precursor	0.021	1.03 (0.094)	1.154 (0.097)	0.164
1,387,208,653	Prothrombin isoform X1	0.038	1.028 (0.093)	1.125 (0.055)	0.130
75,948,172	Coagulation factor II (thrombin)	0.038	1.029 (0.093)	1.125 (0.052)	0.129
296,479,639	Prothrombin	0.038	1.029 (0.093)	1.125 (0.052)	0.129
1,387,237,767	Complement C4 isoform X2	0.029	1.055 (0.058)	0.965 (0.063)	−0.129
1,387,270,779	Pregnancy zone protein isoform X2	0.040	1.234 (0.196)	1.052 (0.166)	−0.230
296,474,001	Phosphatidylinositol-glycan-specific phospholipase D	0.016	1.161 (0.151)	0.972 (0.009)	−0.256
1,351,907	Serum albumin	0.010	1.164 (0.157)	0.973 (0.099)	−0.259
296,486,410	Serum albumin precursor	0.010	1.163 (0.161)	0.971 (0.099)	−0.260
529,482,051	Chain A, Serum albumin	0.010	1.163 (0.161)	0.971 (0.099)	−0.260
295,032,043	Unnamed protein product	0.010	1.171 (0.176)	0.972 (0.099)	−0.269
81,674,813	RBP4 protein	0.009	1.265 (0.287)	0.929 (0.06)	−0.445
31,615,476	Chain A, Plasma Retinol-binding Protein	0.009	1.385 (0.393)	0.952 (0.049)	−0.541
157,831,056	Chain A, Crystallographic Studies On Complexes Between Retinoids And Plasma Retinol-Binding Protein	0.009	1.385 (0.393)	0.952 (0.049)	−0.541

3.2.2. Overlap performance

A significant difference in *γGT* activity between healthy and mastitis cows was observed (Fig. 1A). The healthy cows showed a median value of 4.80 UI/L (range 4.17–8.20; 25-75th percentiles), whereas, in the mastitis group, the activity was significanly higher ($p = 0.002$) with a median of 22.85 UI/L (range 12.30–39.35; 25-75th percentiles) .

3.3. Validation results in SAA

3.3.1. Analytical validation

The intra-assay CVs were 3.9% for the pool with high sAA concentration (mean 94 ± 3.7 SD μg/mL) and 6.3% for the pool with low sAA concentration (mean 5.28 ± 0.3 μg/mL). Additionally, inter-assay CVs were 7.9% for the pool with high sAA concentration (mean 91 ± 7.3 SD μg/mL) and 11.9% for the pool with low sAA concentration (mean 9.2 ± 1.1 SD μg/mL), respectively. Linear regression coefficients of 0.98 and 0.99 were observed when the two saliva samples with high concentrations serially diluted in varying concentrations (1:1 to 1:32) were analysed. The analytical limit of detection calculated was 0.2 μg/mL.

3.3.2. Overlap performance

The SAA concentrations in both groups of healthy and mastitis cows are presented in Fig. 1B. Healthy cows showed significant ($p = 0.0003$) lower SAA concentrations (median: 2.80 μg/mL; range: 2.10–8.55 μg/

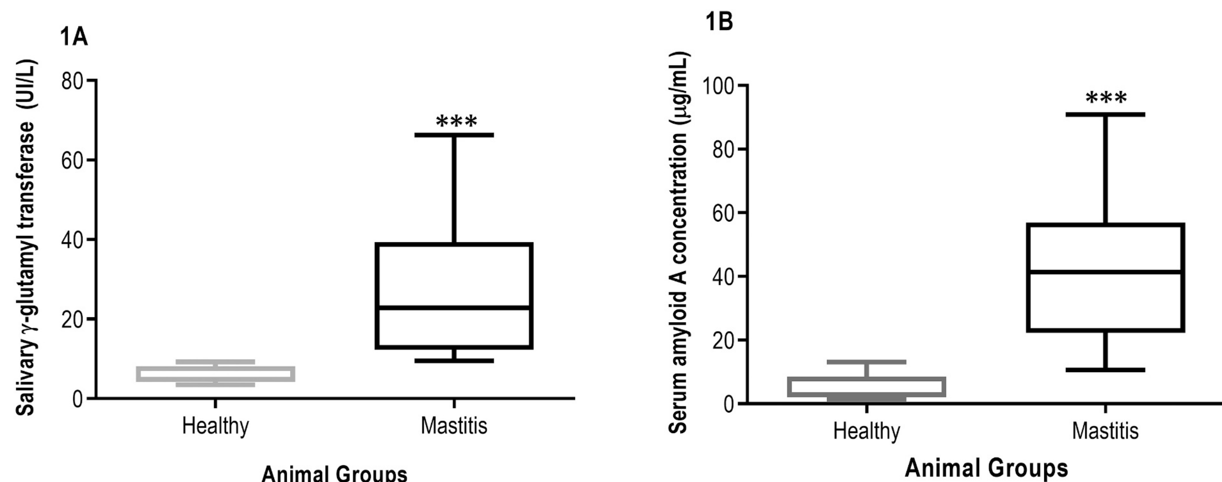


Fig. 1. (A) Salivary γ-glutamyl transferase activity in healthy cows (n = 8) and cow with mastitis (n = 8). (B) Serum Amyloid A concentrations in healthy cows (n = 8) and cow with mastitis (n = 8). The plots show median (line within box), 25th and 75thpercentiles (box) and 5th and 95th percentiles (whiskers). Asterisks indicated significant differences between groups: ***$p < 0.001$.

mL; 25-75th percentiles) in comparison with the mastitis group (median: 41.40 μg/mL; range: 22.28–56.98 μg/mL; 25-75th percentiles).

3.4. Bioinformatics

3.4.1. Saliva

The sixty-three proteins differentially expressed in saliva between healthy and cows with mastitis were used for subsequent bioinformatics analysis in terms of functional clustering, according to the PANTHER classification system, as shown in Fig. 2. The identified differentially modulated proteins in healthy and mastitis had five molecular functions: being binding (GO:0005488) (37.5%, composed by proteins from SERPIN, CAMP, OBP, WAP domain-containing protein (PI3), beta-lactoglobulin (LGB), S100A and PFN1 families), catalytic activity (GO:0003824) (31.3%, SERPIN, PI3, γGT, PIP, WAP four-disulfide core domain protein 18 (WFDC18), PIP, and LYZ C, non-stomach isozyme (LYS)), and molecular function regulators (GO:0098772) (12.5%, SERPINB1, SERPINC1, PI3, and WFDC18) the most represented. A total of eleven different biological processes were highlighted, being cellular process (GO:0009987, composed of SERPINB9, ATIII, γGT, S100A12 and PFN1) and biological regulation (GO:0065007, SERPINB1, SERPINC1, S100A12, PIP and PFN1) (15.6% each one) the most over-represented. The differentially modulated proteins were involved in nine different pathways, being cytoskeletal regulation by Rho GTPase

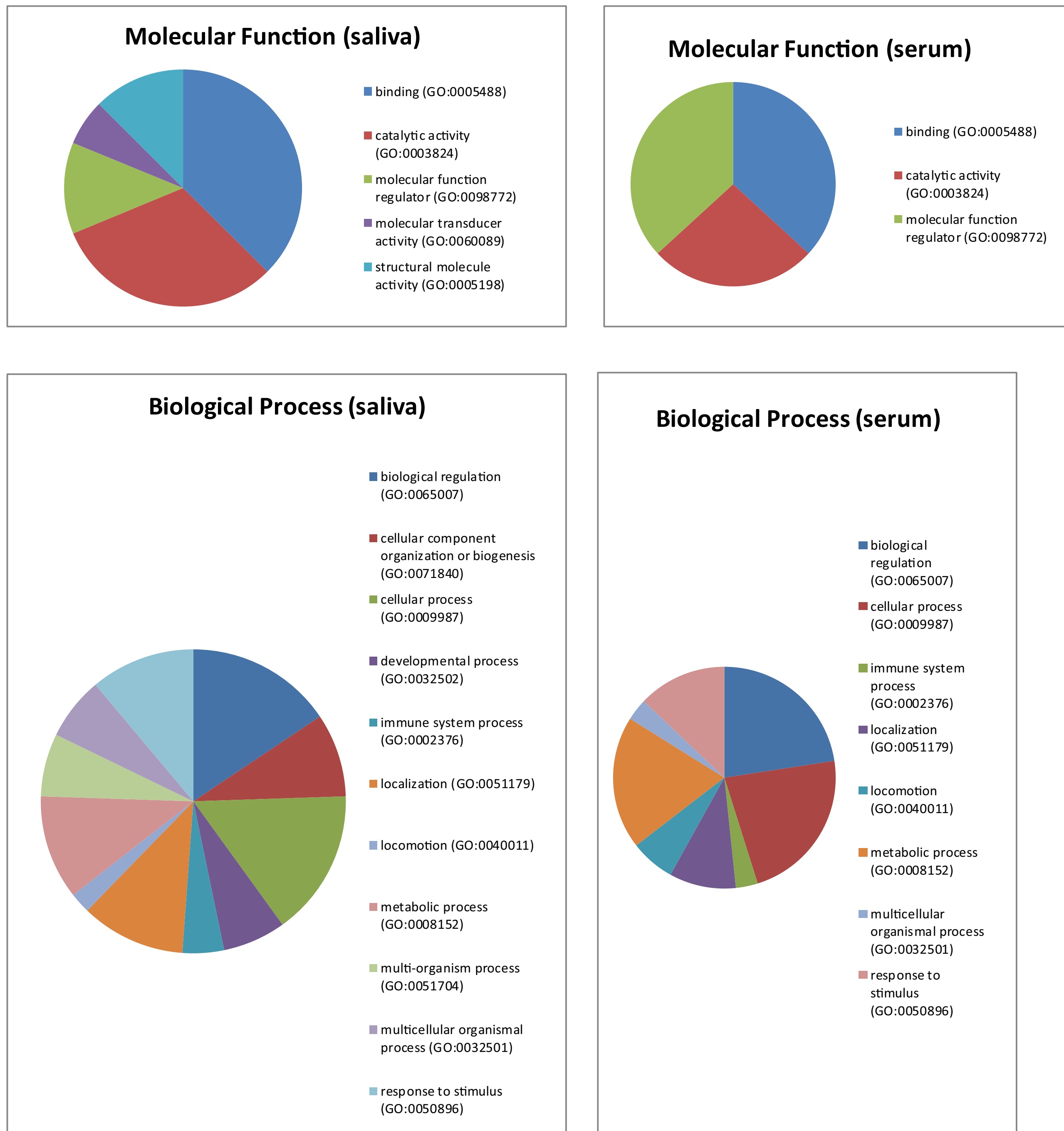


Fig. 2. Molecular function, biological process, pathway, and protein class expressed as a percentage of the total differentially expressed proteins in healthy and mastitis groups in saliva (left) and serum (right), based on the Protein Analysis Through Evolutionary Relationships (PANTHER) classification system (http://www.pantherdb.org/).

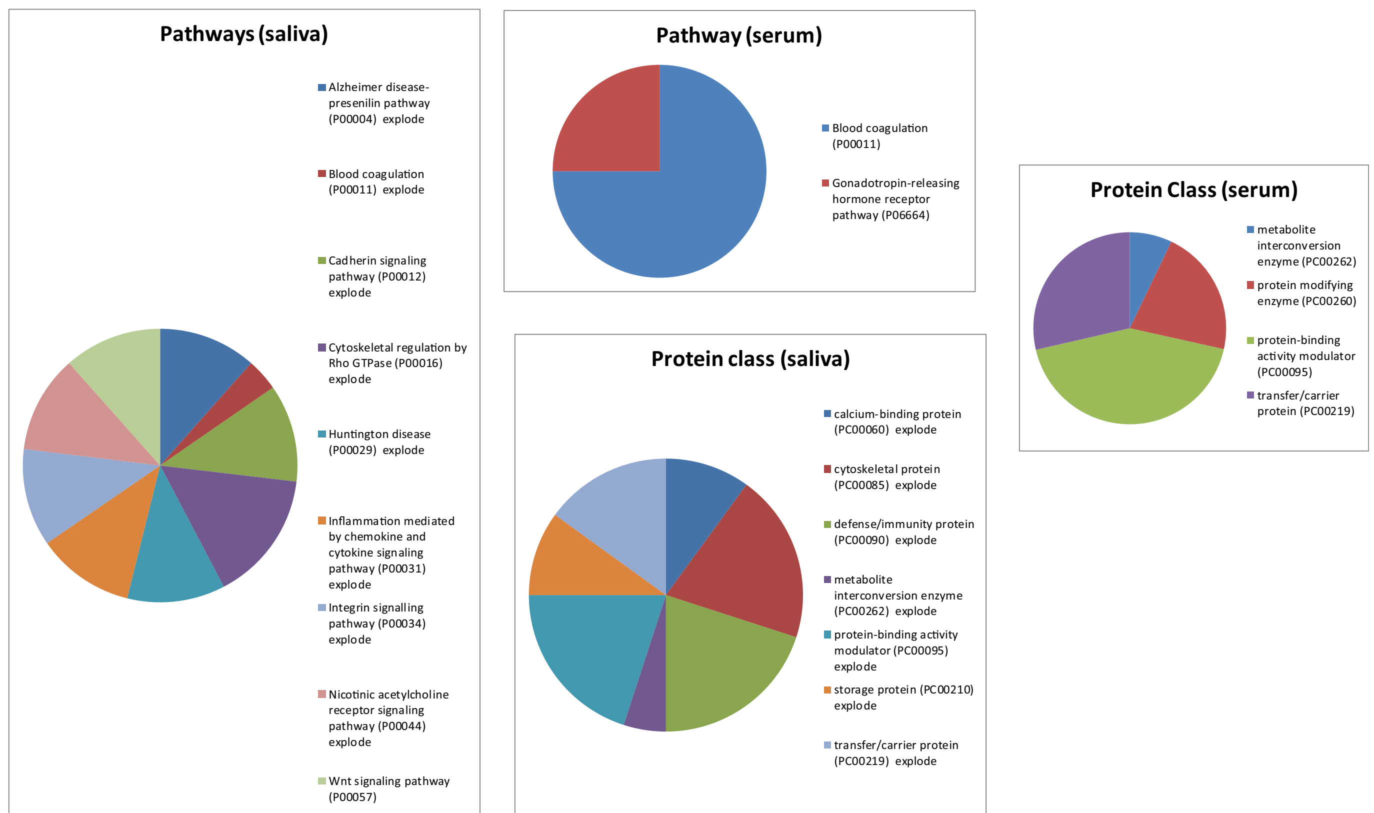


Fig. 2. (*continued*).

the most represented (P00016) (15.4%, composed by ACTN1, actin alpha cardiac muscle 1 (ACTC1), ACTN2 and PFN1), followed by Alzheimer's disease-presenilin pathway (P00004), cadherin signaling pathway (P00012), Huntington disease (P00029), inflammation mediated by chemokine and cytokine signaling (P00031), integrin signaling (P00034), nicotinic acetylcholine receptor signaling (P00044), and Wnt signaling pathways (P00057) (11.5% and composed by proteins from ACTB, ACTC and ACTG1 families in all cases). Finally, blood coagulation (P00011) with a 3.8% (SERPINC1) was the less represented. Regarding protein class, 20% of proteins were protein-binding activity modulator (PC00095, SERPINB1, SERPINC1, PI3 and WFDC18), defense/immunity protein (PC00090, CAMP1, CAMP4, CAMP5, and CAMP6), and cytoskeletal protein (PC00085, ACTB, ACTC, ACTG1). In lesser percentage were observed transfer/carrier protein (PC00219, OBP and LGB) (15%), storage protein (PC00210, beta-casein, CSN2), calcium-binding protein (PC00060, S100A2 and S100A12) (with 10% in both cases), and metabolite interconversion enzyme (PC00262, γGT) (5%).

3.4.2. Serum

The twenty-nine differentially expressed proteins in serum of healthy cows and cows with mastitis were used for subsequent bioinformatics analysis in terms of functional clustering, according to the PANTHER classification system (Fig. 2). The identified differentially modulated proteins in healthy and mastitis had three molecular functions: binding (GO:0005488) (36.8% including RBP4 and eight proteins from the SERPIN A family), molecular function regulators (GO:0098772) (36.8%, being eight proteins from the SERPIN A family), and catalytic activity (GO:0003824) (26.3%, including eight proteins from the SERPIN A family, and F2). Eight different biological processes were involved, being biological regulation (GO:0065007, composed of proteins from SERPIN A family, C4 and F2) and cellular process (GO:0009987, SERPINA and F2) the majoritarian (22.6% each one), followed by metabolic process (GO:0008152) (19.4%, SERPINA and C4) and response to stimulus (GO:0050896) (12.9%, C4 and F2). Blood coagulation was the most represented pathway (P00011) (75%, PROZ and F2), followed by gonadotropin-releasing hormone receptor pathway (P06664) (25%, PI-G PLD). Regarding protein class, 42.9% of proteins were protein-binding activity modulators (PC00095), followed by transfer/carrier proteins (PC00219) (28.6%, including RBP4, SAA and ALB), protein modifying enzyme (PC00260) (21.4%, PROZ and F2), and metabolite interconversion enzyme (PC00262) (7.1%, PI-G PLD).

4. Discussion

In this study, proteome changes in saliva and serum of cows with mastitis by using TMT proteomic analysis are described. To the author's knowledge, this is the first study in which the proteomic changes of saliva in cows with mastitis are reported. The serum was also analysed for comparative purposes. In cows with mastitis, we observed a higher number of proteins showing significant changes in saliva than in serum, and also, the PANTHER analysis showed a higher number of deregulated processes in saliva. None of the proteins differentially modulated in saliva samples matched with the proteins differentially modulated in serum. This discrepancy in the number and types of protein changed between saliva and serum has also been observed in other diseases such as canine pyometra or mammary tumours (Franco-Martínez et al., 2020a, 2020b), and would indicate that both fluids show a different response to the disease and, therefore, can provide with complementary information.

In serum, a total of twenty-nine proteins were found to be deregulated in mastitis cows in comparison to the healthy controls, being nineteen proteins up-regulated and ten down-regulated. Among the proteins up-regulated, the SAA was the protein with the highest difference of expression, but also isoforms of serpin A3, immunoglobulins and components of the blood-clotting mechanism such as prothrombin or thrombin were differentially modulated. The increased levels of serpin A3 and immunoglobulins may be explained as an attempt of the animal to overcome a bacterial infection and were in line with previous findings

(Alonso-Fauste et al., 2012; Turk et al., 2012). The changes in coagulation parameters are also a frequent finding in dairy cows with clinical mastitis (Ismail and Dickinson, 2010). Among the down-regulated proteins, there were serum RBP and albumin. The RBP is a protein involved in retinol transport (vitamin A), and it has been reported to decrease in plasma and milk in cows due to inflammation (Van Merris et al., 2004; Abd Eldaim et al., 2010). This decrease could be attributed to an increase in vitamin A consumption in various tissues during the inflammatory process (Kanda et al., 1990). Albumin is a negative acute-phase protein and decreases in cows have been associated with increases in immunoglobulins since albumin synthesis in the liver is expected to decrease to favour globulin production (Bertoni et al., 2008; Bobbo et al., 2017).

In our proteomic analysis and the immunoturbidimetric assay tested to validate the proteomic results, the cows with mastitis showed higher serum SAA levels. SAA is a major acute-phase protein and a well-established biomarker of inflammation in ruminants (Eckersall et al., 2001). The immunoturbidimetric assays have the advantage of providing an automated and fast measurement of this acute-phase protein. Recently, a report using the same immunoturbidimetric assay as our study found increases in SAA in the serum of cows with mastitis, having this protein a higher sensitivity and specificity than other acute-phase proteins, such as haptoglobin or α-1 acid glycoprotein (Otsuka et al., 2020). Overall, the increase of SAA in serum of cows with mastitis confirms the validity of our experimental model.

In saliva, a total of sixty-three proteins were deregulated in mastitis when compared to healthy controls, being forty-five proteins up-regulated and eighteen down-regulated. Among the proteins up-regulated, salivary proteins that exert host immune defense functions with antimicrobial function and are essential components of the immune response such as short palate (Wei et al., 2014), lysozyme (Callewaert and Michiels, 2010), or S100 calcium-binding protein (Zackular et al., 2015) were found. Other proteins that were up-regulated in mastitis were beta-lactoglobulin and beta-casein. Increases in these proteins have been previously described in milk of cows with mastitis (Mansor et al., 2013; Thomas et al., 2016). Among the identified down-regulated proteins, the cathelicidins are antimicrobial peptides with a role in the innate immune system of many vertebrates and are stored in neutrophil and macrophage granules (Kościuczuk et al., 2012). Decreases in cathelicidins and also in actin, which was another protein down-regulated in our study, have been described before in milk of cows with mastitis (Abdelmegid et al., 2018).

The protein selected for our validation study in saliva was γGT since it showed significant differences between the two groups in the proteomic analyses and can be measured by an easy-to-set-up spectrophotometric assay. In a proteomic study, Van Altena et al. (2016) identified the γGT in milk as a potential biomarker that could be used to indicate dairy cows at risk to develop health problems. However, the γGT milk levels could not be quantified in that study due to the lack of sensitivity of the immunoassay employed. In our case, the γGT activity in saliva samples of both healthy and mastitis cows showed levels higher than the limit of detection of the assay used for the validation of the proteomic results, indicating that the assay was enough sensitive to detect the activity of this enzyme in saliva. In addition, our analytical validation demonstrated that this automated assay showed good accuracy (coefficient of correlation close to one), and precision for the γGT activity measurements in cow saliva samples. Our overlap performance results showed a significant increase of 4.76-fold in γGT activity in the median values of cows with mastitis compared to healthy cows. Although further studies with a higher number of animals are needed, our results constitute the first report of the potential possible usefulness of γGT levels in saliva as a biomarker of mastitis in cows. Although the mechanisms of the increases of γGT in mastitis should be elucidated, they could be related to the immune system activation since it has been demonstrated that T lymphocytes express γGT and this expression is increased on activated T cells (Henson et al., 1999), as occurs in mastitis (Souza et al., 2020). In another species as the horse, significant increases of salivary γGT activity in an inflammatory process as in acute abdominal disease have been observed (Contreras-Aguilar et al., 2019a, 2019b).

The present study has some limitations. First, a low number of animals were included in the validation study, so these results should be further confirmed in larger cohorts. In addition, it should be evaluated if mastitis caused by other pathogens such as *E. coli*, *S. aureus* or *Mycoplasma spp* produces similar proteome findings since, for example, changes of different magnitude have been described in acute-phase proteins in milk with mastitis caused by different types of pathogens (Dalanezi et al., 2020) and, in our study, the type of bacteria not was added to the statistical model. For the selection of the protein we have considered significant fold changes with $p < 0.05$, independently than fold change value itself. This is because (1) there is still not defined a specific cut-off in the literature for the fold change and different ranges of fold change are used; (2) by considering as relevant only changes greater or lower than a cutoff could introduce a serious bias in the results. This is because there are proteins that exert important biological functions and in which a small change can cause important modifications in the intracellular signals transmission cascade, as reported before in other studies (Xiao et al., 2016). However, if we defined a cut-off point of 1.2 fold change that would equal of 0.230 to −0.230 log2 of fold change, all saliva proteins reported to be significantly different in our study, except one (WFDC18) showed also significant changes.

In conclusion, there are changes in protein expression and metabolic pathways in the saliva and serum proteomes of cows with mastitis, showing different response patterns. Besides, some of these proteins showing changes can be measured using commercially available assays that can be automatized and thus, could be considered as potential biomarkers of mastitis. However, the results of this study must be confirmed with different pathogens causing mastitis to become associated with classic test for mastitis.

Ethics statement

In this experiment only used surplus samples collected before in article Contreras et al., (2019b) and the study protocol was approved by the Lithuanian National Commission of Ethics (No. license G2–60, 2017-02-15).

Declaration of Competing Interest

The authors have declared that no competing interests exist.

Acknowledgement

Seneca Foundation of Murcia Region (grant number: 19849/GERM/15) funded this research. The proteomic analysis was supported partly by the European Regional Development Fund (Grant Agreement KK.01.1.1.04.0086). In addition, this work was supported by a help of mobility "Moving Minds CMN" of the Murcia University within the framework to carry out research and technical training stays for PDI and PAS in foreign canters (R-27/2020). LFM and MD·C-A. were granted by the predoctoral contract "FPU" by the University of Murcia (Spain), grant number R-605/2016. AMP was funded by a post-doctoral grant (21239/P.D./19. Fundación Séneca, Región de Murcia, Spain). DE has a post-doctoral fellowships granted by the contract "Convocatoria relevo generacional para el fomento de la investigación" by the University of Murcia (Spain), grant number R-1.437/2019.

References

Abd Eldaim, M.A., Kamikawa, A., Soliman, M., Ahmed, M.M., Okamatsu-Ogura, Y., Akira, T., Miyamoto, T., Kimura, K., 2010. Retinol binding protein 4 in dairy cows: its presence in colostrum and alteration in plasma during fasting, inflammation, and the peripartum period. J. Dairy Res. 77, 27–32.

Abdelmegid, S., Murugaiyan, J., Abo-Ismail, M., Caswell, J.L., Kelton, D., Kirby, G.M., 2018. Identification of host defense-related proteins using label-free quantitative proteomic analysis of Milk whey from cows with Staphylococcus aureus subclinical mastitis. Int. J. Mol. Sci. 19 (1), 78.
Almeida, A.M., Bassols, A., Bendixen, E., Bhide, M., Ceciliani, F., Cristobal, S., Eckersall, P.D., Hollung, K., Lisacek, F., Mazzucchelli, G., McLaughlin, M., Miller, I., Nally, J.E., Plowman, J., Renaut, J., Rodrigues, P., Roncada, P., Staric, J., Turk, R., 2015. Animal board invited review: advances in proteomics for animal and food sciences. Animal 9, 1–17.
Alonso-Fauste, I., Andrés, M., Iturralde, M., Lampreave, F., Gallart, J., Alava, M.A., 2012. Proteomic characterization by 2-DE in bovine serum and whey from healthy and mastitis affected farm animals. J. Proteome 75, 3015–3030.
Ang, C.S., Binos, S., Knight, M.I., Moate, P.J., Cocks, B.G., McDonagh, M.B., 2011. Global survey of the bovine salivary proteome: integrating multidimensional prefractionation, targeted, and glycocapture strategies. J. Proteome Res. 10, 5059–5069.
Baeumlisberger, D., Arrey, T.N., Rietschel, B., Rohmer, M., Papasotiriou, D.G., Mueller, B., Beckhaus, T., Karas, M., 2010. Labeling elastase digests with TMT: informational gain by identification of poorly detectable peptides with MALDI-TOF/TOF mass spectrometry. Proteomics 10, 3905–3909.
Bertoni, G., Trevisi, E., Han, X., Bionaz, M., 2008. Effects of inflammatory conditions on liver activity in puerperium period and consequences for performance in dairy cows. J. Dairy Sci. 91, 3300–3310.
Bobbo, T., Fiore, E., Gianesella, M., Morgante, M., Gallo, L., Ruegg, P.L., Bittante, G., Cecchinato, A., 2017. Variation in blood serum proteins and association with somatic cell count in dairy cattle from multi-breed herds. Animal 11, 1–11.
Callewaert, L., Michiels, C.W., 2010. Lysozymes in the animal kingdom. J. Biosci. 35, 127–160.
Contreras-Aguilar, M.D., Escribano, D., Martínez-Subiela, S., Martín-Cuervo, M., Lamy, E., Tecles, F., Cerón, J.J., 2019a. Changes in saliva analytes in equine acute abdominal disease: a sialochemistry approach. BMC Vet. Res. 15 (1), 187.
Contreras-Aguilar, M.D., Monkeviciene, I., Ceron, J.J., Silinskas, I., Vallejo-Mateo, P.J., Tecles, F., Martinez-Subiela, S., Tvarijonaviciute, A., Zelvyte, R., 2019b. Biochemical changes in saliva of cows with inflammation: a pilot study. Res. Vet. Sci. 124, 383–386.
Contreras-Aguilar, M.D., Vallejo-Mateo, P.J., Želvyte, R., Tecles, F., Peres Rubio, C., 2020. Changes in saliva analytes associated with lameness in cows: a pilot study. Res. Vet. Sci. 124, 383–386. Animals (Basel) 9;10(11):2078.
Dalanezi, F.M., Schmidt, E.M.S., Joaquim, S.F., Guimarães, F.F., Guerra, S.T., Lopes, B.C., Cerri, R.L.A., Chadwick, C., Langoni, H., 2020. Concentrations of acute-phase proteins in Milk from cows with clinical mastitis caused by different pathogens. Pathogens 9 (9), 706.
Dayon, L., Turck, N., Garci-Berrocoso, T., Walter, N., Burkhard, P.R., Vilalta, A., Sahuquillo, J., Montaner, J., Sanchez, J.C., 2011. Brain extracellular fluid protein changes in acute stroke patients. J. Proteome Res. 10, 1043–1051.
Eckersall, P.D., Young, F.J., McComb, C., Hogarth, C.J., Safi, S., Weber, A., McDonald, T., Nolan, A.M., Fitzpatrick, J.L., 2001. Acute phase proteins in serum and milk from dairy cows with clinical mastitis. The Veter. Rec. 148, 35–41.
Escribano, D., Horvatić, A., Contreras-Aguilar, M.D., Guillemin, N., Cerón, J.J., Tecles, F., Martinez-Miró, S., Eckersall, P.D., Manteca, X., Mrljak, V., 2019a. Changes in saliva proteins in two conditions of compromised welfare in pigs: an experimental induced stress by nose snaring and lameness. Res. Vet. Sci. 125, 227–234.
Escribano, D., Horvatić, A., Contreras-Aguilar, M.D., Guillemin, N., Cerón, J.J., Lopez-Arjona, M., Hevia, M.L., Eckersall, P.D., Manteca, X., Mrljak, V., 2019b. Identification of possible new salivary biomarkers of stress in sheep using a high-resolution quantitative proteomic technique. The Res. Veter. Sci. 124, 338–345.
Franco-Martínez, L., Horvatić, A., Gelemanović, A., Samardžija, M., Mrljak, V., Contreras-Aguilar, M.D., Martínez-Subiela, S., Dąbrowski, R., Tvarijonaviciute, A., 2020a. Changes in the salivary proteome associated with canine Pyometra. Front. Veter. Sci. 11 (7), 277.
Franco-Martínez, L., Gelemanović, A., Horvatić, A., Contreras-Aguilar, M.D., Dabrowski, R., Mrljak, V., Cerón, J.J., Martínez-Subiela, S., Tvarijonaviciute, A., 2020b. Changes in serum and salivary proteins in canine mammary tumors. Animals 10 (4), 741.
Giese, H., Dilly, M., Gundelach, Y., Hoffmann, G., Schmicke, M., 2018. Influence of transrectal palpation training on cortisol levels and heart rate variability in cows. Theriogenology 119, 238–244.
Giron, P., Dayon, L., Turck, N., Hoogland, C., Sanchez, J.C., 2011. Quantitative analysis of human cerebrospinal fluid proteins using a combination of cysteine tagging and amine-reactive isobaric labeling. J. Proteome Res. 10, 249–258.
Halasa, T., Huijps, K., Østerås, O., Hogeveen, H., 2007. Economic effects of bovine mastitis and mastitis management: a review. The Vetinary Quart. 29, 18–31.
Henson, S.H., Nichols, T.C., Holers, V.M., Karp, D.R., 1999. The ectoenzyme gamma-glutamyl transpeptidase regulates antiproliferative effects of S-nitrosoglutathione on human T and B lymphocytes. J. Immunol. 163, 1845–1852.
Horvatić, A., Guillemin, N., Kaab, H., McKeegan, D., O'Reilly, E., Bain, M., Kuleš, J., Eckersall, P.D., 2018. Integrated dataset on acute phase protein response in chicken challenged with Escherichia coli lipopolysaccharide endotoxin. Data Brief 17, 684–699.
Ismail, Z.A., Dickinson, C., 2010. Alterations in coagulation parameters in dairy cows affected with acute mastitis caused by E. coli and S. aureus pathogens. Vet. Res. Commun. 34, 533–539.
Kanda, Y., Yamamoto, N., Yoshino, Y., 1990. Utilization of vitamin a in rats with inflammation. Biochim. Biophys. Acta 1034, 337–341.
Kościuczuk, E.M., Lisowski, P., Jarczak, J., Strzałkowska, N., Jóźwik, A., Horbańczuk, J., Krzyżewski, J., Zwierzchowski, L., Bagnicka, E., 2012. Cathelicidins: family of antimicrobial peptides. A review. Mol. Biol. Rep. 39, 10957–10970.
Lamy, E., Mau, M., 2012. Saliva proteomics as an emerging, non-invasive tool to study livestock physiology, nutrition and diseases. J. Proteome 75, 4251–4258.
Mallikarjunappa, S., Adnane, M., Cormican, P., Karrow, N.A., Meade, K.G., 2019. Characterization of the bovine salivary gland transcriptome associated with *Mycobacterium avium* subsp. *paratuberculosis* experimental challenge. BMC Genomics 20 (1), 491.
Mansor, R., Mullen, W., Albalat, A., Zerefos, P., Mischak, H., Barrett, D.C., Biggs, A., Eckersall, P.D., 2013. A peptidomic approach to biomarker discovery for bovine mastitis. J. Proteome 85, 89–98.
Martinez-Subiela, S., Horvatic, A., Escribano, D., Pardo-Marin, L., Kocaturk, M., Mrljak, V., Burchmore, R., Ceron, J.J., Yilmaz, Z., 2017. Identification of novel biomarkers for treatment monitoring in canine leishmaniosis by high-resolution quantitative proteomic analysis. Vet. Immunol. Immunopathol. 191, 60–67.
Otsuka, M., Sugiyama, M., Ito, T., Tsukano, K., Oikawa, S., Kazuyuki Suzuki, K., 2020. Diagnostic utility of measuring serum amyloid a with a latex agglutination turbidimetric immunoassay in bovine mastitis: comparison with haptoglobin and alpha 1 acid glycoprotein. J. Vet. Med. Sci. 29, 20–0550.
Souza, F.N., Blagitz, M.G., Batista, C.F., Takano, P.V., Gargano, R.G., Diniz, S.A., Silva, M.X., Ferronatto, J.A., Santos, K.R., Heinemann, M.B., De Vliegher, S., Della Libera, A.M.M.P., 2020. Immune response in nonspecific mastitis: what can it tell us? J. Dairy Sci. 103, 5376–5386.
Thomas, F.C., Mullen, W., Tassi, R., Ramírez-Torres, A., Mudaliar, M., McNeilly, T.N., Zadoks, R.N., Burchmore, R., David, Eckersall, 2016. 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. bioSystem 12, 2735–2747.
Turk, R., Piras, C., Kovačić, M., Marko Samardžija, M., Ahmed, H., De Canio, M., Urbani, A., Meštrić, Z.F., Soggiu, A., Bonizzi, L., Roncada, P., 2012. Proteomics of inflammatory and oxidative stress response in cows with subclinical and clinical mastitis. J. Proteome 75, 4412–4428.
van Altena, S.C.E., de Klerk, B., Hettingac, K.A., van Neervena, R.J.J., Boerene, S., Savelkoula, H.F.J., Tijhaar, E.J., 2016. A proteomics-based identification of putative biomarkers for diseasein bovine milk. Vet. Immunol. Immunopathol. 174, 11–18.
van Merris, V., Meyer, E., Duchateau, L., Blum, J., Burvenich, C., 2004. All-trans retinoic acid is increased in the acute phase-related hyporetinemia during Escherichia coli mastitis. J. Dairy Sci. 87, 980–987.
Vissio, C., Dieser, S.A., Agnelli, H.L., Odierno, L.M., Larriestra, A.J., 2014. Accuracy of the composite somatic cell count to detect intra-mammary infection in dairy cows using latent class analysis. Prev. Veter. Med. 113, 547–555.
Wei, Y., Xia, W., Ye, X., Fan, Y., Shi, J., Wen, W., Yang, P., Li, H., 2014. The antimicrobial protein short palate, lung, and nasal epithelium clone 1 (SPLUNC1) is differentially modulated in eosinophilic and noneosinophilic chronic rhinosinusitis with nasal polyps. J. Allergy Clin. Immunol. 133, 420–428.
Xiao, H., Zhang, Y., Kim, Y., Sung, Kim, Kim, J.J., Kim, K.M., Yoshizawa, J., Fan, L.-Y., Cao, C.-X., Wong, D.T.W., 2016. Differential proteomic analysis of human saliva using tandem mass tags quantification for gastric cancer detection. Sci. Report. 25 (6), 22165.
Zackular, J.P., Chazin, W.J., Skaar, E.P., 2015. Nutritional immunity: S100 proteins at the host-pathogen Interface. J. Biol. Chem. 290 (31), 18991–18998.